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A study of matrix metalloproteinases in cancer and atherosclerosis

by

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Abstract

Background: Matrix metalloproteinases (MMPs) have been shown to be involved in cancers and atherosclerosis, the leading causes of present day mortality. The objectives of the cancer element of this project were to investigate single nucleotide polymorphisms (SNPs) in MMP1 and MMP8 regarding breast cancer and malignant melanoma, and a functional characterisation of the genetic variants, including the MMP1 polymorphism rs19799750, previously associated with multiple cancers. The objective of the second part of this project was to investigate whether MMP8 played a role in the development of atherosclerotic lesions and if so, the underlying mechanisms.

Methods/Results: Genetic investigations found the MMP8 SNP rs11225395 to be associated with the occurrence of both breast cancer and malignant melanoma; furthermore it was also associated with reduced lymph node metastasis, reduced cancer relapse and greater survival. Functional luciferase assays showed that the minor allele of the polymorphism has higher promoter activity in breast cancer and melanoma cell lines. They also showed haplotypic effects on MMP1 promoter activity in several cancer cell lines by the 2G allele of polymorphism rs1799750 and one or more MMP1 promoter SNPS. The second part of the study found an association between a MMP8 SNP and the extent of coronary atherosclerosis; additionally a relationship among MMP8 gene variation, plasma VCAM-1 level, and atherosclerosis progression was observed in a prospective study. Murine studies showed reduced atherosclerosis in MMP8/ApoE knockout mice compared with ApoE knockout littermate controls. Biochemical studies confirmed that MMP8 can convert angiotensin I to angiotensin II.

Conclusions: The data of the first part of this project support the notion that genetic polymorphisms in the MMP1 and MMP8 influence the expression of these genes and the development and progression of cancer. The results of the second part of this project indicate an important role of MMP8 in the pathogenesis of atherosclerosis.

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Contents

1.0 Introduction

1.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs), also known as matrixins are secreted zinc-dependent endopeptidases. There are 24 known MMP genes in humans belonging to the metzincin superfamily of metallopeptidases (2, 3). Members of the metzincin superfamily, consisting of astacins, seralycins, ADAMs (a disintegrin and metalloprotease)/adamalysins, and MMPs, are characterised by their topologically similar and conserved zinc-binding catalytic domains (4). As a family MMPs are capable of degrading nearly all the constituents of the extracellular matrix (ECM) and consequently play a vital role in many physiological processes such as embryogenesis, tissue remodelling, wound healing and angiogenesis; they are also involved in many pathological conditions including osteoarthritis, autoimmune diseases, cardiovascular diseases and cancer (5). The complexity of MMPs' involvement in physiological processes and disease is underlined by the fact that they not only degrade components of the ECM but also act on non-structural substrates; cleaving/shedding cell bound growth factors and receptors, adhesion molecules, cytokines and many more bio-active compounds (6).

1.1.1 MMP structure

The classic structure of MMPs consists of a signal peptide domain, propeptide, catalytic domain, hinge region, and hemopexin domain; depending upon the MMP other domains/features may or may not be added, such as a fibronectin domain, vitronectin

insert, type I transmembrane domain, type II transmembrane domain, glycosylphosphatidylinositol (GPI) anchor, cytoplasmic domain, cysteine array region, IgG-like domain, or a furin cleavage site (1, 7).

As mentioned earlier, the zinc-binding motif within the catalytic site of MMPs is highly conserved, as is the cysteine switch motif within the propeptide region. In the catalytic site there are three histidine (H) residues and a glutamate (E) that make up the principal features of the zinc-binding motif HEXXHXXGXXH (X indicates any amino acid). The histidine residues serve to coordinate the zinc ion, while the glutamate acts as a nucleophile during the hydrolysis of peptide bonds. There is also a highly conserved methionine (M) residue within a structure known as a "Met-turn", a polypeptide chain that loops beneath the zinc ion allowing the methionine to act as a hydrophobic base for the binding site (2, 4, 7, 8). The cysteine switch motif and mechanism will be discussed later.

1.1.2 MMP classification

MMPs have traditionally been classified via their substrate specificity and structural domains. The subgroups have been categorised as the collagenases, gelatinases, matrilysins, furin-activated secreted MMPs, transmembrane-type MMPs, GPI-anchored MT-MMPs, type II transmembrane MMPs, and other MMPs (figure 1.1).

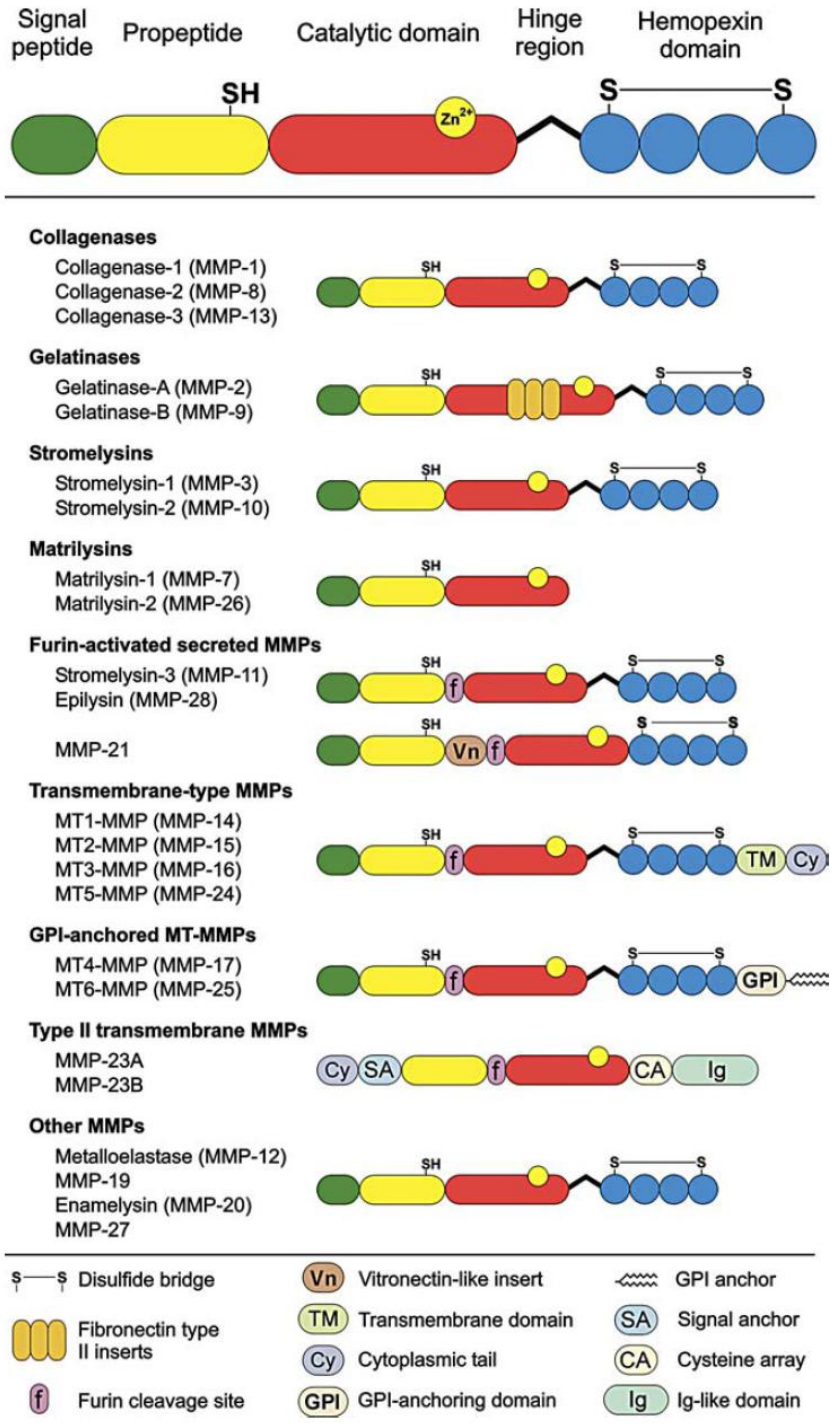

Figure 1.1. Human MMPs classified into subgroups via their substrates specificities and structural domains. Taken from (1).

1.1.3 Regulation of MMPs

1.1.3.1 Transcriptional regulation of MMPs

The majority of MMPs are not constitutively expressed, but expressed at certain times such as in physiological or pathophysiological tissue remodelling (9). Transcriptional regulation plays a key role in the control of expression of MMPs; it can occur by several different mechanisms including the epigenetic processes of acetylation and methylation, and the action of transcription factors on *cis*-elements within gene promoter regions.

The principal method of regulation of transcription of MMPs is probably, by the action of transcription factors on *cis*-elements within gene promoter regions, this allows controlled cell specific expression. The majority of MMPs have *cis*-elements in common with other members of the family. Response elements that have been identified as important in the regulation of MMPs are response elements for activator protein-1 (AP-1), polyomavirus enhancer-A binding protein-3 (PEA-3), nuclear factor kappa B (NFκB), and the signal transducers and activators of transcription (STAT) (3).

Acetylation, in this case a reversible modification of histone proteins, loosens the interaction between DNA and histone within the chromatin complex. Loosening allows transcription factors access to DNA and mostly promotes the activation of genes. Histones are acetylated by a group of proteins called histone acetyl transferases (HATs); deacetylation is via a group of proteins called histone deacetylases (HDACs). The fact that inhibitors of HDACs have been shown to both repress and augment the expression of induced MMPs suggests that their regulation through the mechanism of acetylation is

mainly via its effects on the genes of signalling molecules upstream of MMPs in pathways leading to their expression (6).

Methylation of CpG islands within the promoter regions of eukaryotic genes, carried out by methyl transferases, in the main acts as a negative regulation mechanism, repressing gene expression. Methylation of cytosine bases can hinder the binding of certain transcription factors to recognition sequences; additionally methylated DNA binding proteins may recruit co-repressor molecules that inhibit gene expression (10). Some MMPs have been shown to be partly regulated by methylation status, there was an inverse relationship between MMP9 promoter methylation and its expression in lymphoma cells (11), also the knockout of methyl transferases in colon cancer cells lead to an increase in expression of MMP3 (12).

1.1.3.2 Post-transcriptional regulation of MMPs

MMPs may also be regulated via post transcriptional mechanisms. Stabilisation of messenger RNA (mRNA) enhances the expression of genes; mRNA may be stabilised by the action of trans-acting factors interacting with *cis*-elements within mRNA. MMP1 and MMP3 have been shown to have their mRNA stabilised, and expression enhanced, via the interaction of p38 MAPK with AU rich elements (AREs) within their 3' untranslated regions (UTR) in fibroblasts (13). More recently it was demonstrated that MMP-16 is down-regulated by microRNA-146b in glioma (14).

1.1.3.3 Activation of zymogens

Nearly all MMPs, with the exception of type I & II transmembrane MMPs, are secreted zymogens, requiring the removal of the propeptide before becoming fully active. Enzyme activity is blocked with the propeptide via a cysteine switch mechanism, in which a cysteine residue within a conserved part of the propeptide forms a covalent bond to the zinc ion in the catalytic site. All the methods of MMP activation such as treatment with organomercurials, sodium dodecyl sulphate, or other proteases involve the dissociation of the cysteine residue from the zinc ion thereby activating the catalytic site, and the subsequent autolysis of the propeptide domain (15).

1.1.3.4 Inhibition of MMP activity by TIMPs

The major method of protein regulation of MMPs is via tissue inhibitors of metalloproteinases (TIMPs); which as a family are capable of inhibiting all the MMPs. There are 4 members of the TIMP family numbered 1-4. Factors effecting the regulation of MMPs by TIMPs are varying efficacy against specific MMPs allied with differential tissue expression and localisation. Inhibition occurs on a 1:1 ratio with the TIMP protein binding to the zinc ion of the catalytic site preventing any protease activity by the MMP (1). In the case of MMP8, its form expressed on the surface of polymorphonuclear cells is highly resistant to inhibition by TIMPs (16)

1.1.3.5 Polymorphisms and MMP gene regulation

Naturally occurring sequence variations such as single nucleotide polymorphisms (SNPs) are prevalent throughout the genome; they are variants common across the population and may occur anywhere within or in the area surrounding a gene (17). The vast majority of SNPs appear to be neutral, at present, having no discernible effect on any biological processes. However some SNPs have been shown to exert allele specific differences over a biological process, sometimes having an association with disease. Functional effects can occur if a SNP changes the coding sequence for a gene bringing about an amino acid change in the sequence of a protein, this can alter its structure, activity and function. Changes, by SNPs, to *cis*-elements in the promoter region, or to AREs in UTRs may affect *trans*-acting proteins interaction with DNA and subsequently alter gene expression; other changes may be to splice sites or even at loci relatively distant to genes that effect DNA folding changes. Not all SNPs of interest will cause functional effects but can be biomarkers if in strong linkage disequilibrium (LD) with undiscovered functional SNPs. The concept of LD was eloquently summed up by Goldstein & Weale in 2001 (18); "*alleles at different loci are sometimes found together more or less often than expected based on their frequencies. In population genetics, this non-random pattern is called linkage disequilibrium*". Several polymorphisms within the promoters of MMPs have been shown to affect their gene expression and be associated with disease; the first such polymorphism in MMPs to be described was the 5A/6A variant in the promoter of MMP3; linked to coronary atherosclerosis (19, 20). It has subsequently shown to be situated within an NFκB responsive element (21). Multiple SNPs within gene promoters may act in concert to exert haplotypic effects on expression as studies on interleukin 6 (IL-6) and cholesteryl ester transfer protein (CETP) show (22, 23). Colleagues have shown evidence haplotypic effects on MMP1

expression and the risk of myocardial infarction (24). Haplotypes for the purposes of this study can be defined as multiple SNPs occurring on the same chromosome that are (in relative terms) commonly inherited together. A brief overview of MMP polymorphisms in disease is shown in table 1.1, adapted from (3).

Table 1.1 Functional SNPs in MMP promoters

Gene	Polymorphism	rsID	Promoter Activity	Associated pathology	
				Tumour-disease	Non-tumour disease
MMP1	-1607 1G/2G	rs1799750	Higher	↑ Risk: lung [1] and colorectal [2] cancer, Poor prognosis: breast [3] and ovarian [4] cancer, cutaneous malignant melanoma [5]	↑ Risk: idiopathic pulmonary fibrosis [6], Poor prognosis: cirrhosis [7]
MMP2	−1575 G/A	rs243866	Lower	Breast cancer [8] (in vitro MCF-7 cells)	
	−1306 C/T	rs243865	Lower	↓ Risk: oesophageal [9] and lung [10] cancer, gastric cardia adenocarcinoma [11], oral squamous cell carcinoma [12]	↓ Risk: lumbar disc disease [13]
	−735 C/T	rs2285053	Lower	↓ Risk: oesophageal [9] and lung [10] cancer	
MMP3	−1171 5A/6A	rs3025058	Lower	↓ Risk: lung [14] breast [15] and oral [16] cancer	↑ Risk: atherosclerosis [17], Poor prognosis: rheumatoid arthritis [18]
MMP7	−181 A/G	rs11568818	Higher	↑ Risk: gastric [19], cervical [20] and oral [21] cancer, oesophageal squamous cell carcinoma and non-small cell lung carcinoma [22]	Poor prognosis: atherosclerosis [23]
MMP8	−799 C/T	rs11225395	Higher	Better prognosis: breast cancer [24]	↑ Risk: preterm premature rupture of membranes (haplotype) [26]
	−381 A/G	rs1320632	Higher		↑ Risk: preterm premature rupture of membranes (haplotype) [26]
	+17 C/G	rs2155052	Higher	↓ Risk: lung cancer [25]	↑ Risk: preterm premature rupture of membranes (haplotype) [26]
MMP9	−1562 C/T	rs3918242	Higher	↑ Risk: oral cancer [27]	↑ Risk: atherosclerosis [28, 29], abdominal aortic aneurysm [30]
MMP12	−82 A/G	rs2276109	Lower	Poor prognosis: bladder cancer [31]	

[1] (25), [2] (26), [3] (27), [4] (28), [5] (29), [6] (30), [7] (31), [8] (32), [9] (33), [10] (34), [11] (35), [12] (36), [13] (37), [14] (38), [15] (39), [16] ,(40) [17] (41), [18] (42), [19] (43), [20] (44), [21] (45), [22] (46), [23] (47), [24] (48), [25] (49), [26] (50), [27] (51), [28] (52), [29] (53), [30] (54), [31] (55).

1.1.4 MMP Genes

1.1.4.1 Location and homology

In humans 9 of the 24 MMP genes occur within a cluster at chromosome 11q22, the other genes are widely spread across 10 chromosomes (table 1.2).

Table 1.2 Chromosomal locations of human MMP genes and the site of their highest detected expression

MMP Gene	Chromosomal Location	Highest human expression via GeneAtlas*
MMP23a	1p36.3	-
MMP23b	1p36.3	Heart
MMP16	8q21	Cardiomyocytes
MMP21	10q26.13	Skeletal muscle
MMP26	11p15	Skeletal muscle
MMP7	11q22.3	Pancreatic islet
MMP20	11q22.3	Cardiomyocytes
MMP27	11q22.3	Cardiomyocytes
MMP8	11q22.3	Bone marrow
MMP10	11q22.3	Uterus corpus
MMP1	11q22.3	Smooth muscle cells
MMP3	11q22.3	Smooth muscle cells
MMP12	11q22.3	Tonsil
MMP13	11q22.3	Cardiomyocytes
MMP19	12q14	Adipocyte
MMP17	12q24.3	Prefrontal cortex
MMP14	14q11-12	Smooth muscle cells
MMP25	16p13.3	Skeletal muscle
MMP2	16q12	Smooth muscle cells
MMP15	16q21	Thyroid
MMP28	17q12	Lung
MMP24	20q11.2	Cerebellum penduncles
MMP9	20q13.2	Bone marrow
MMP11	22q11	Placenta

*Site of highest expression levels in humans, as detected by GeneAtlas (56) via the BioGPS portal (57).

Despite the relative diversity of functions and characteristics of the MMP family, there is considerable inter-species homology shown between vertebrates; evidence for the

ancient origins of MMPs is given by the presence of many orthologue genes in the invertebrate *Ciona intestinalis* (58). The homology of MMPs between vertebrates has been taken advantage of in order to further elucidate the biological roles of these proteases, particularly using murine models. Of mice and men; 23 have been discovered in mice and 24 MMPs discovered in humans. There is a duplication of MMP1 specific to mice, and a gain of MMP26 and the duplication of MMP23 specific to humans (3).

1.1.4.2 MMP expression

As mentioned earlier most MMPs are not constitutively expressed, but expressed at discrete times within physiological or pathophysiological processes. This occurs at times and sites that require cell migration or degradation of the ECM such as embryogenesis, tissue remodelling, wound healing and angiogenesis; including osteoarthritis, autoimmune diseases, cardiovascular diseases and cancer (5). Tissue sites with the highest detected mRNA expression for the respective MMP genes are listed in table 1.2, the data was obtained from the GeneAtlas project (56) via the BioGPS portal (57).

1.1.5 Biochemistry and substrates of MMPs

It is commonly stated that MMPs as a family are capable of degrading nearly all the constituents of the ECM. However their influence is not merely limited to its structural modification; sequestered within the ECM are signalling molecules such as growth factors which may be released along with collagen fragments with cryptic matrix signals (59). Non-structural and non-matrix proteins are also processed, not limited to cell

bound growth factors and receptors, adhesion molecules, cytokines and other MMP zymogens (6).

1.1.5 Substrates of MMP1 and MMP8

The two MMPs at the centre of this work are MMP1 and MMP8; both are collagenases, also known as collagenase 1 and 2 respectively. The collagenases are the principal enzymes that cleave the native fibrillar collagens, types I, II, and III; with the hemopexin domain of collagenases being intrinsic to their ability to bind to and cleave the triple helix structure (60). MMP1 shows preference to cleave type III over type I and II, while MMP8 has preferential activity against type I, then type II, then type III; MMP8 seems to be the most potent of all the collagenases against types I and II collagens (61-63). The known substrates of MMPs 1 and 8 are shown in table 1.3, adapted from (1) and (64).

Table 1.3. Known substrates of MMP1 and MMP8

MMP	Collagenous substrates	Other substrates
MMP-1 (Collagenase-1)	aggrecan, collagens III> I>II, VII, VIII, X, XI	AAT, ACT, α2M, casein, C1q, fibrin, fibrinogen, IL-1β, MCP-1, -3, -4, proTNF-α, proMMP-1, proMMP-2, nidogen, fibronectin, MBP, gelatin, IGFBPs, laminin, link protein, perlecan, tenascin, vitronectin
MMP-8 (Collagenase-2)	aggrecan, Collagen type I > type II > type III, Collagen VII, Collagen X,	AAT, α2M, C1q, fibrinogen, substance P, proMMP-8, tachykinin, angiotensin, IL-8*, LIX, MIG, IP-10, MCP-1α**, IGFBP-5, L-selectin, Laminin-5, nidogen, fibronectin, tenascin

* Tester, 2007
** Low-efficiency cleavage by human MMP-8, and no cleavage by murine MMP-8.

1.1.6 Role of MMPs in physiological and pathophysiological processes

As noted previously MMPs, through their modulation of cell-ECM interactions, influence cell differentiation, signalling and migration and are involved in embryogenesis, tissue remodelling, wound healing and angiogenesis. They are also involved in many pathological conditions including osteoarthritis, autoimmune diseases, cardiovascular diseases and cancer (5). A selection MMPs found to be associated with disease are shown in table 1.1.

1.1.6.1 Roles of MMP1 and MMP8 in physiological processes

Examples of physiological situations in which MMP1 is expressed are in the skin during embryonic development and in basal keratinocytes in wound healing (65, 66). MMP8 is expressed in maturing neutrophils within the bone marrow, then sequestered in granules for later action, and is also expressed in articular chondrocytes within the cartilage (67, 68).

1.1.6.2 Roles of MMP1 and MMP8 in pathophysiological processes

MMP1 has been shown to be expressed in chronic cutaneous ulcers (1); it has also been implicated in the weakening of fibrous caps in atherosclerosis increasing the chances of plaque rupture, and is expressed in different cell types within atherosclerotic lesions (69-71). The majority of studies on MMP1 and disease involve cancer, these are discussed elsewhere. MMP8 is implicated in bronchitis and rheumatoid arthritis (1); the involvement of MMP8 in cancer and atherosclerosis is also discussed elsewhere.

1.2 Cancer

1.2.1 The impact of cancer on the population

Cancer is the term given to a disease caused by the loss of normal growth regulation and tissue organisation; these losses can result in the formation of a tumour or neoplasm. In all cancers this abnormal growth and regulation is a direct consequence of changes to the genetic composition of the cell; be it the loss, mutation, addition or translocation of genetic material. The common risk factors of cancer all involve substances or processes that can lead to genetic changes; such as smoking, exposure to ultra violet (UV) or ionising radiation, or exposure to mutagenic chemicals. Malignant cancers are characterised by their ability to spread to and invade other tissues away from the original site of neoplastic transformation. Cancer is one of the leading causes of mortality globally today, currently 1 in 4 deaths in the United States and 1 in 8 worldwide are due to cancer (72, 73).

1.2.2 Hallmarks of cancer

The hallmarks of cancer, as characterised by Hanahan & Weinberg (74), are self sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

Cancers can become self sufficient in growth signals by manufacturing their own thereby producing autocrine stimulation; examples of this are the production of tumour

growth factor α (TGFα) by sarcomas and platelet-derived growth factor (PDGF) by glioblastomas (75).

One of the best described methods of cancer becoming insensitive to antigrowth factors is via the transforming growth factor-β (TGF-β) pathway with TGF-β receptors being down-regulated in many cancers (76). TGF-β can halt advancement from the G1 phase of the cell cycle the suppression of the c-myc gene, a cell cycle regulator, or by the blocking of cyclin:cyclin dependent kinase complexes responsible for phosphorylation of the retinoblastoma protein (pRb).

The avoidance of programmed cell death, apoptosis, is an intrinsic function of cancers to tumour survival and successful invasion of naive tissues; one of the many anti-cancer activities of the tumour suppressor protein p53 is the upregulation of the pro-apoptotic Bax protein in response to DNA damage. The p53 tumour suppressor gene is found mutated in over 50% of all human tumours (77). Normal cells are able to replicate a finite amount of times, 60 -70 in observations of cultured cells. The counting mechanism for cell generations involves telomeres, thousands of hexamer repeats found at the ends of chromosomes. Telomeres shorten each successive generation; when they are lost the resulting chromosomal instability inevitably leads to cell death. Cancer cells acquire seemingly limitless replicative potential via maintenance of telomeres. Telomerase is an enzyme which adds hexamer repeats to the end of chromosomes that is found unregulated in 85 – 90% of cancers (78).

Sustained angiogenesis is an important part of tumour development, tumours requiring increased oxygen and nutrients as they grow. The pro-angiogenic vascular endothelial

growth factor (VEGF) is found to be unregulated in tumours; drugs inhibiting VEGF or its receptor VEGFR have proven successful in reducing neovascularisation and tumour growth in animal studies. Thrombospondin-1, an inhibitor of angiogenesis, is positively regulated by p53 in some cell types. Therefore loss of function to p53 can result in lower levels of thrombospondin-1 and consequently less thrombospondin-1 dependent anti-angiogenic effects (79).

1.2.3 Metastasis

A succinct description of this phase of cancer is given in the review by Gupta & Massague, "Metastasis occurs when genetically unstable cancer cells adapt to a tissue microenvironment that is distant from the primary tumour". Its importance is underlined by the fact it accounts for 90% of the mortality attributed to solid cancer (80). The steps (shown in figure 1.2, taken from Fidler *et al. 2003* (81)) from the initial neoplasm (**a**) towards metastasis are the proliferation and vascularisation of the primary tumour (**b**), detachment of cells from the primary tumour and invasion of the vasculature (lymphatics, venules, and capillaries) (**c**), circulation and aggregation (through recruitment of platelets and/or lymphocytes), adherence to vessel walls (**d**), extravasation and adaptation of or adaptation to a microenvironment (**e**), and finally further proliferation and angiogenesis (**f**) to establish the metastasis at the distant site.

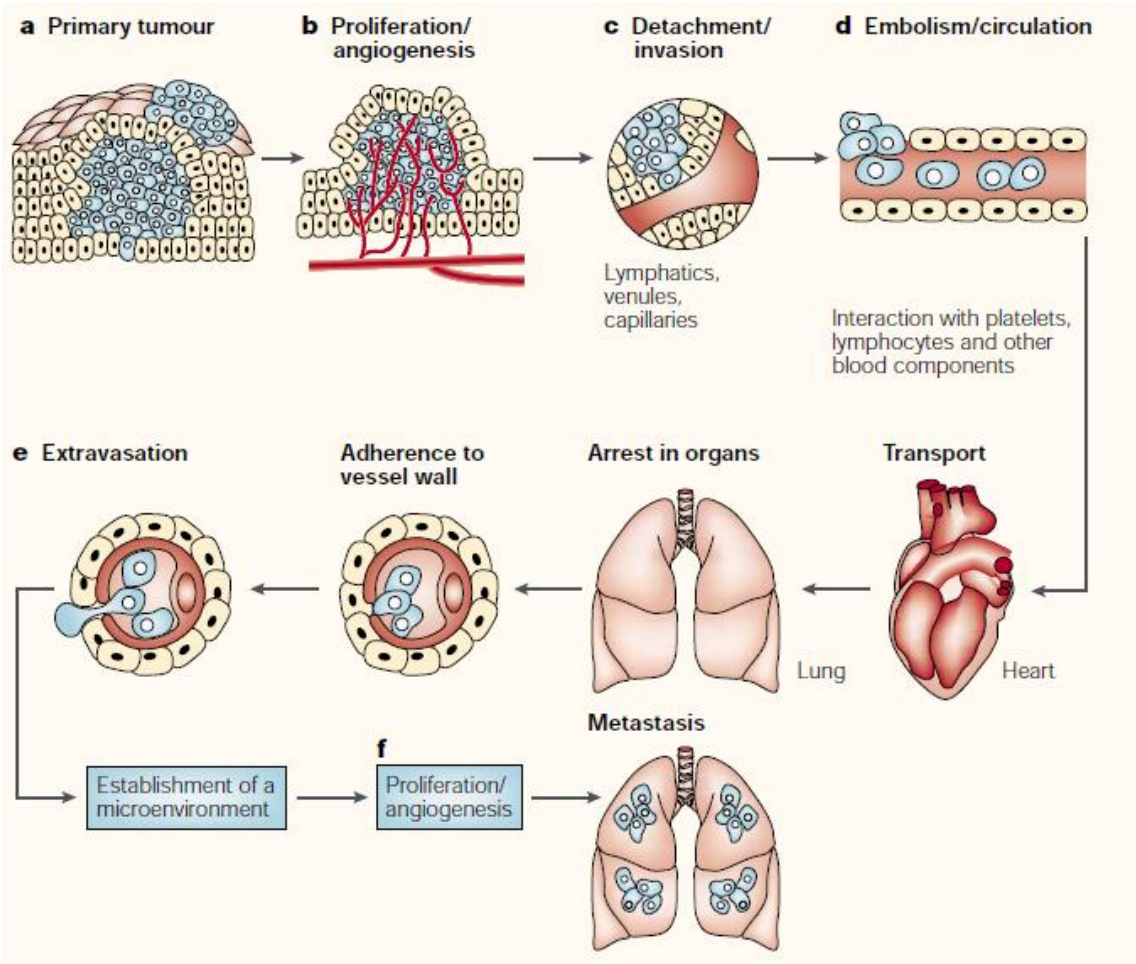

Figure 1.2. The main steps in the formation of a metastasis (81).

1.2.3.1 Metastatic cell movement

The entry of tumour cells into the vasculature (intravasation) is an important and potentially limiting stage of metastasis. It involves the movement of cells from the primary tumour through the ECM and then access to the circulation through pores in the basement membrane/endothelium. The fastest observed movement of carcinoma cells is when they move along linear paths provided by collagen containing fibres of the ECM. The speed is thought to be facilitated by the forming of short lived focal complexes with the fibres; the cells exhibit solitary amoeboid motion and shape also giving them the ability to squeeze through dense ECM. Imaging studies on the

differences between non metastatic and metastatic cell lines derived from the same mammary tumour show cells from the metastatic cell line (MTLn3) to linearly move along ECM fibres while cells from the non metastatic cell line (MTC) do not. MTLn3 cells show amoeboid movement and are not polarised except when in the presence of epidermal growth factor (EGF) like ligands attracting them to blood vessels, MTC cells are generally more polarised but exhibit no chemotaxis to EGF-like ligands and thus blood vessels; MTC cells showed notable fragmentation on the exposure of their pseudopods to the shear forces of blood flow, MTLn3 cells, expressing higher levels of cytokeratins, had greater mechanical strength enabling them to survive this last step of intravasation (82).

1.2.4 Breast Cancer

One of the principal cancers investigated in this work, in relation to MMPs, is breast cancer; it has the highest rate of incidence and is the second highest cause of cancer mortality (15%) amongst females in the United States (72). It has been well established that breast cancer has a strong familial association; women with first degree relatives with the disease have approximately double the risk of incidence of breast cancer compared to the general population (83). Mutations to highly penetrant tumour suppressor genes BRCA1 and BRCA2 only account for about 20% of the two fold increased risk to close female relatives of breast cancer patients; searches for other high penetrance genes and novel foci have failed account for the majority of familial risk, suggesting it may be conferred by multiple polygenic low penetrance foci (84). Described previously in section 1.2.3.1 metastatic breast cancer cells have been

observed, in vivo, to move rapidly along ECM fibres within primary tumours exhibiting solitary amoeboid movement also enabling them to pass through dense networks of matrix that could retard non-amoeboid cells. They have been seen to only show strong polarisation when in the presence of EGF-like ligands indicating blood vessels; the chemotactic cytokines may be produced by the high numbers of extremely motile macrophages and other leukocytes visualised by intravital imaging in the vicinity of blood vessels of mammary tumours (82).

1.2.4.1 Prognostic markers of breast cancer

As with other solid cancers, metastasis is the principal cause of death in breast cancer. Prognostic markers currently in clinical use, relating to breast cancer metastasis, are described below (table 1.4, adapted from (85)).

Table 1.4. Breast cancer metastasis prognostic markers

Marker	Use in clinic	Metastatic determinants	Details
Tumour size	Established	Tumours under 2 cm in diameter have a low risk of metastasis; tumours of 2–5 cm have a high risk of metastasis; tumours over 5 cm have a very high risk of metastasis	Independent prognosis marker
Axillary lymph node status	Established	If there are no lymph-node metastases, the risk of metastasis is low; if lymph-node metastases are present, the risk of metastasis is high; the presence of over 4 lymph-node metastases is associated with very high metastasis risk	Related to tumour size
Histological grade	Established	Grade 1 tumours have a low risk of metastasis; grade 2 tumours have an intermediate risk of metastasis; grade 3 tumours have a high risk of metastasis	Related to tumour size
Angioinvasion	Established in patients with lymph-node negative tumours	The presence of tumour emboli in over 3 blood vessels is associated with metastasis	In patients with lymph-node negative tumours
uPA/PAI1 protein level	Newly established marker	High protein levels of uPA and PAI1 are associated with high metastasis risk	Independent prognosis marker
Steroid-receptor expression	Established for adjuvant therapy decision	Low steroid-receptor levels are associated with metastasis	Short-term predictor of metastasis risk (5 years); related to histological grade
ERBB2 gene amplification and protein expression	Established for adjuvant therapy decision	*ERBB2* amplification/over expression is associated with metastasis	In patients with lymph-node positive tumours
Gene-expression profiling	Recently established	A ‘good signature’ of 70 genes is associated with low metastasis risk; a ‘poor signature’ of 70 genes is associated with high metastasis risk	Tested in patients with lymph-node negative tumours

1.2.5 Malignant melanoma

Melanoma represents the most deadly form of skin cancer. The incidence of this aggressive cancer is increasing rapidly. Skin cancer when including basal and squamous cell carcinoma is the most common neoplasm in the United States. However melanoma, while comprising of approximately 4% of skin cancer is responsible for 80% of the attributed deaths of skin cancer. Common risk factors for melanoma are a previous family history multiple and/or atypical naevi. Environmental factors include immunosuppression, exposure to UV radiation and skin sensitive to sunlight (86, 87). The median survival rate of malignant melanoma (MM) is 8-9 months, the 3 year overall survival (OS) is less than 15% (88). Early diagnosis of melanoma enables it to be cured by surgical resection. The majority of melanomas are cured this way. Once metastasis occurs the disease is extremely resistant to current therapies (89).

The cancer itself is derived from melanocytes, cells which are responsible for the pigmentation of the skin. The process of transformation of melanocytes into a malignant disease can be said to take place in four stages. Initially in normal skin, melanocytes are evenly distributed. Moles or ‘naevi’ occur at foci with increased numbers of melanocytes; these are considered as benign. Naevi may subsequently undergo a radial-growth phase (RGP), changing shape, size, and/or colour; this is considered to a premalignant stage. The next stage is termed the vertical-growth-phase (VGP), the tumour invades the dermis and subcutaneous tissue; the possibility of invasion of the lymphatic and vascular systems means this stage is the first considered to have malignant potential and can lead directly to metastasis (89, 90). Characteristic of the VGP is the expression of basement membrane and matrix degrading enzymes, such as

MMPs that may facilitate invasion through the dermis; MMP1 in particular has been implicated as an important player in this phase (90-92).

Molecular studies have indicated that amongst the more common melanomas the gene most frequently mutated is the serine–threonine protein kinase B-RAF (BRAF). Another commonly mutated gene that is mutually exclusive of BRAF is NRAS (93). Table 1.5 adapted from (89), highlights the more common genetic alterations found in MM.

Table 1.5. Common genetic alterations found in malignant melanoma

Gene type	Gene	Alteration frequency/type(s) in melanoma (%)	Reference
Oncogenes	BRAF	50–70% mutated	(94)
	NRAS	15–30% mutated	(94, 95)
	AKT3	Overexpressed	(96)
Tumour suppressors	CDKN2A	30–70% deleted, mutated or silenced	(97)
	PTEN	5–20% deleted or mutated	(95, 98)
	APAF-1	40% silenced	(99)
	P53	10% lost or mutated	(100)
Others	Cyclin D1	6–44% amplified	(101)
	MITF	10–16% amplified	(101)

Abbreviations: serine–threonine protein kinase B-RAF (BRAF), Neuroblastoma RAS viral oncogene homolog (NRAS), V-akt murine thymoma viral oncogene homolog 3 (AKT3), Cyclin-dependent kinase inhibitor 2A (CDKN2A), Phosphatase and tensin homolog (PTEN), Apoptotic protease activating factor 1 (APAF-1), tumour protein 53 (p53), Microphthalmia-associated transcription factor (MITF)

1.2.5 MMPs and cancer

The prevailing scientific doctrine regarding MMPs and cancer for many years was that tumour-associated MMPs provided a route for metastasis by degrading the surrounding ECM thereby granting access to the vasculature and lymphatic systems; this was backed up by various studies showing the upregulation of several MMPs to be associated with multiple types of neoplastic disease. However subsequent clinical trials for broad spectrum MMP inhibitors as anti-cancer therapeutics yielded poor and sometimes adverse results (102). Further evidence has since come to light of a much more complex role in both physiological and disease situations, in cancer not all MMPs are detrimental, some MMPs have been shown to have a protective effect while others are either pro-tumourigenic or protective depending on the situation (103). MMP2 is an example of an MMP that to date seems to be only pro-tumourigenic; a promoter haplotype (rs243865-C, rs2285053-C) that shows increased activity in luciferase vectors, increased mRNA expression, and interaction with a DNA binding protein, confers increased risk of oesophageal cancer and metastasis, and increased risk of lung cancer (33, 34). The C allele of rs243865 (present in the aforementioned haplotype) has also been shown to confer increased risk of gastric cardia adenocarcinoma and oral squamous cell carcinoma (35, 36). MMP9 when co-localised with CD44, a cell surface proteoglycan, increases tumour cell invasiveness (104). However it can also play a protective role; expression levels of MMP9 are inversely correlated with liver metastasis in colorectal cancer, and low levels of MMP9 caused increased tumour vascularisation in mice (103, 105). MMP12 is thought to have a largely beneficial effect against cancer; murine studies indicate MMP12 expression decreased tumour growth rates (106), and in human subjects homozygote carriers of the low expressing allele of a promoter polymorphism (rs2276109) had increased risk of bladder cancer metastasis

(55). Further examples of MMPs - their polymorphisms and their effects on cancers can be found in table 1.1.

1.2.5.1 MMP1 and cancer

The presence or high levels of expression of MMP1 is associated with a poor prognosis in many types of cancer, including colorectal cancer, oesophageal cancer, pancreatic ductal adenocarcinoma, breast cancer, and melanoma (107-111). One promoter variant in particular is shown to influence expression; a guanine (G) insertion (-1607; rs1799750) creates a binding site for the E-twenty six (Ets) transcription factor that contributes to increased transcription (112). Subsequent investigations show the 2G allele, with augmented expression of MMP1 has been associated with increased risk lung and colorectal cancers and a poorer prognosis with breast cancer, ovarian cancer and melanoma (25-29, 113-116).

1.2.5.2 MMP8 and cancer

The role of MMP8 in tumour progression is less straightforward; it has been detected in vivo in head and neck small cell squamous carcinomas (117), and it has been implicated with and associated with the invasiveness of ovarian cancer (118, 119). Other studies give an anti-tumour role to MMP8; one group found that expression levels of MMP8 inversely correlated with pro-metastatic behaviour in isogenic breast cancer cell lines (120), the same group, following up their previous work, were able to engineer altered metastatic phenotypes in the two cell lines by changing the MMP8 expression; by knocking down MMP8 in the non-metastatic cell line to give it an invasive phenotype,

conversely upregulating MMP8 in the metastatic cell line to give it a non-invasive phenotype (121). Researchers based in Spain found that MMP8 knockout mice have increased susceptibility to chemically induced skin carcinogenesis (122).

1.3 Atherosclerosis

Atherosclerosis is a progressive condition often termed hardening or furring of the arteries. It is a chronic inflammatory disease, which occurs over decades, although serious acute clinical symptoms brought about by this disease can manifest in minutes. It is the principal contributing factor for both cardiovascular disease (CVD) and cerebrovascular disease; combined they are the largest cause of death on the planet today (123, 124). In Europe the latest available data puts the cause of death for men at 21% for coronary heart disease (CHD), 11% for stroke, and 11% for other cardiovascular disease; for women the figures are 22% for CHD, stroke 17%, and 15% for other CVD (125). As diet and lifestyle of world becomes more westernised the epidemic cardiovascular disease and associated mortality is spreading to more undeveloped countries.

1.3.1 Clinical manifestations of atherosclerosis

1.3.1.1 Angina Pectoris

Angina pectoris is severe pain in the chest caused by ischaemia of the myocardium. This is mainly due to obstruction of coronary vessels supplying the heart by atherosclerotic plaques, although it can occur as a result of vasospasm. There are two subtypes of angina pectoris termed stable and unstable.

Stable angina is most common subtype; sufferers typically experience chest discomfort that lasts several minutes, which will have been precipitated by increased activity and

oxygen demand. The discomfort reflects atherosclerosis advanced enough to restrict maximal blood flow during exercise (126).

Unstable angina (UA) is the more grave condition and is also a subtype of acute coronary syndrome (acute coronary syndrome covers unstable angina, non-ST segment elevation myocardial infarction (NSTEMI) and ST segment elevation myocardial infarction (STEMI). Sufferers of UA may experience increased discomfort for prolonged periods (over 15 minutes). Symptoms can occur unpredictably even at rest or following minimal activity; the severity, frequency and duration of these symptoms increase over time. The mechanism of this condition is plaque rupture and subsequent thrombotic events (127); without medical intervention the possibility of myocardial infarction is extremely high.

1.3.1.2 Acute Myocardial Infarction

Myocardial infarction (MI) more commonly known as 'heart attack' is classified, under acute coronary syndrome, into two further subtypes, NSTEMI and STEMI. These subtypes are named for their appearance on an electrocardiogram (ECG/EKG) (128). Pathologically MI is defined as myocardial cell death due to prolonged ischaemia (129). Myocardial infarction is the single largest cause of death in the world today (130), once again the major underlying cause is coronary heart disease/atherosclerosis; plaque rupture and subsequent thrombosis leading to occlusion of coronary vessels. Extended lack of blood and oxygen to the affected heart muscle causes necrosis and apoptosis in the cardiomyocytes.

1.3.1.3 Stroke

When the blood supply to part of the brain is cut or reduced the resulting loss of neurological function is termed ‘stroke’. Short term ischaemia may only produce reversible loss of function, prolonged ischaemia leads to infarction, irreversible cell death in the affected brain tissue. Brain damage caused by stroke can lead to physical and/or mental impairment, and death, particularly if the region of the brain affected controls the respiratory or cardiovascular system (131).

1.3.2 Risk factors of atherosclerosis

Common systemic risk factors strongly associated with atherosclerosis are hypercholesterolaemia, hypertension, smoking and diabetes; a closer look at the factors involved in the aetiology of atherosclerosis is given in table 1.6, taken directly from the review by Lusis in 2000 (132).

Table 1.6 Genetic and environmental factors associated with atherosclerosis and coronary heart disease (CHD) (132)

Factors with a strong genetic component	**Comments**
Elevated levels of LDL/VLDL	Associations demonstrated in epidemiological studies and supported by studies of genetic disorders and animal models. Clinical trials have shown benefits of cholesterol reduction.
Reduced levels of HDL	Associations demonstrated by numerous epidemiological studies and supported by studies of genetic diseases and animal models.
Elevated levels of lipoprotein(a)	Associations observed in many, but not all, epidemiological studies. Animal studies have been contradictory.
Elevated blood pressure	Associations observed in epidemiological studies. Clinical trials have demonstrated benefits of blood pressure reduction, with particularly strong effects on stroke.
Elevated levels of homocysteine	Associations have been observed in epidemiological studies, and homocystinuria results in severe occlusive vascular disease.
Family history	When all known risk factors are controlled for, family history remains a very significant independent factor.
Diabetes and obesity	Associations observed in epidemiological studies and in studies with animal models.
Elevated levels of haemostatic factors	Significant independent associations have been observed with elevated levels of fibrinogen, plasminogen activator inhibitor type 1 and platelet reactivity.
Depression and other behavioural traits	Associations observed in several population studies.
Gender (male)	Below age 60, men develop CHD at more than twice the rate of women.
Systemic inflammation	Elevated levels of inflammatory molecules such as C-reactive protein are associated with CHD, as are inflammatory diseases such as rheumatoid arthritis.
Metabolic syndrome	This cluster of metabolic disturbances, with insulin resistance as a central feature, is strongly associated with CHD.
Environmental factors	
High-fat diet	Population migration and epidemiological studies indicate strong associations with lifestyle, and diet appears to be the most significant factor. High-fat, high-cholesterol diets are usually required for development of atherosclerosis in experimental animals.
Smoking	Strong associations observed in numerous epidemiological studies. Clinical trials have demonstrated the benefit of stopping smoking.
Low antioxidant levels	Results of clinical trials with antioxidants have not been conclusive. Fat-soluble antioxidants protect against atherosclerosis in experimental animals, however.
Lack of exercise	Significant independent associations with CHD.
Infectious agents	Epidemiological studies provide suggestive evidence for associations with various infectious agents, such as *Chlamydia pneumoniae*. Preliminary animal studies support the relationship.

1.3.3 Sites of lesion development

Atherosclerosis is characterised by development of lesions/plaques in large to medium sized arterial vessels (132). The lesions develop at specific locations within the vessels that experience low or oscillatory shear stress such as those proximal to branches, bifurcations or within highly curved arteries (133). A clue as to why atheromas develop at these sites may be given by the architecture of the endothelium; in areas of laminar flow endothelial cells show a regular elliptical morphology and orientation giving a tightly packed formation. Areas experiencing low or oscillatory shear stress cells have a more polygonal shape with little uniform orientation and a less dense formation. It is these areas of non-uniform endothelial cells which have increased permeability to large molecules such as low density lipoprotein (LDL) that are much more vulnerable to plaque (134, 135).

1.3.4 Growth of atheroma

The first signs of atherosclerosis are small fatty streaks made up almost entirely of lipid laden macrophages and T-lymphocytes; this earliest form of the disease can commonly be found in infants and young children. In this initial stage of the disease progression occurs in the form of expansion of the lesions both laterally and vertically with adjacent lesions coalescing. Plaque formation follows with the recruitment of smooth muscle cells from the intima and deposition of a collagen matrix to form a fibrous cap (136). It is easy to think of the above process as a smooth continuum occurring throughout life of a patient with the chronic condition. Angiographic studies however point to discontinuous stenosis and plaque development.

Three types of events have been put forward for these bursts in the expansion of atheromatous lesions. Within atherosclerotic lesions microvasculature develops in response to pro-angiogenic molecules secreted by macrophages, such as fibroblast growth factors, vascular endothelial growth factor (VEGF), and certain MMPs. It aids lesion growth by supplying nutrients but critically these vessels are fragile and susceptible to haemorrhage leading to production of thrombin. Further lesion development occurs when the in situ thrombin activates platelets to release platelet-derived growth factor (PDGF), stimulating SMC migration and proliferation, and transforming growth factor beta (TGF-β) which in turn stimulates SMCs to produce interstitial collagen. The most important event in terms of plaque growth and clinical outcomes is the rupture of the fibrous cap. This type of rupture is responsible for approximately 75% of acute Myocardial infarctions, but in all probability most of these ruptures remain sub-clinical. The release of previously sequestered tissue factor and other thrombogenic material may often lead incomplete occlusion of the afflicted vasculature; circulating thrombolytic agents enable the thrombus to be re-absorbed. The re-absorption process as with microvascular haemorrhage involves the build up of SMCs and collagen deposition; this can advance fatty streaks to fibrous plaques and/or lead more regions of the adjacent vasculature to become likely sites of atherogenesis. The final case is the erosion of small areas of the endothelial monolayer; in areas affected by erosion von Willebrand factor and collagen can be exposed; these molecules provide an aggregatory stimulus for platelet thrombus formation. The majority of these events are sub-clinical although this process may account for nearly 25% of fatal thromboses (123).

1.3.5 Two types of plaque

Atherosclerotic plaques can be divided into two categories, stable and unstable. Stable plaques have a thick fibrous cap; they can still cause clinical problems related to stenosis of the arterial vessels, but they are much less likely to rupture leading to acute clinical events. Unstable plaques are characterised by thin fibrous caps and increased levels of MMPs and are much more prone to rupture. If the fibrous cap then ruptures thrombogenic materials can be released, such as lipids and tissue factor. The resulting thrombus formation can lead to the occlusion of vessels, leading to myocardial infarction and/or stroke (123, 132, 137).

1.3.6 Formation of an atherosclerotic plaque

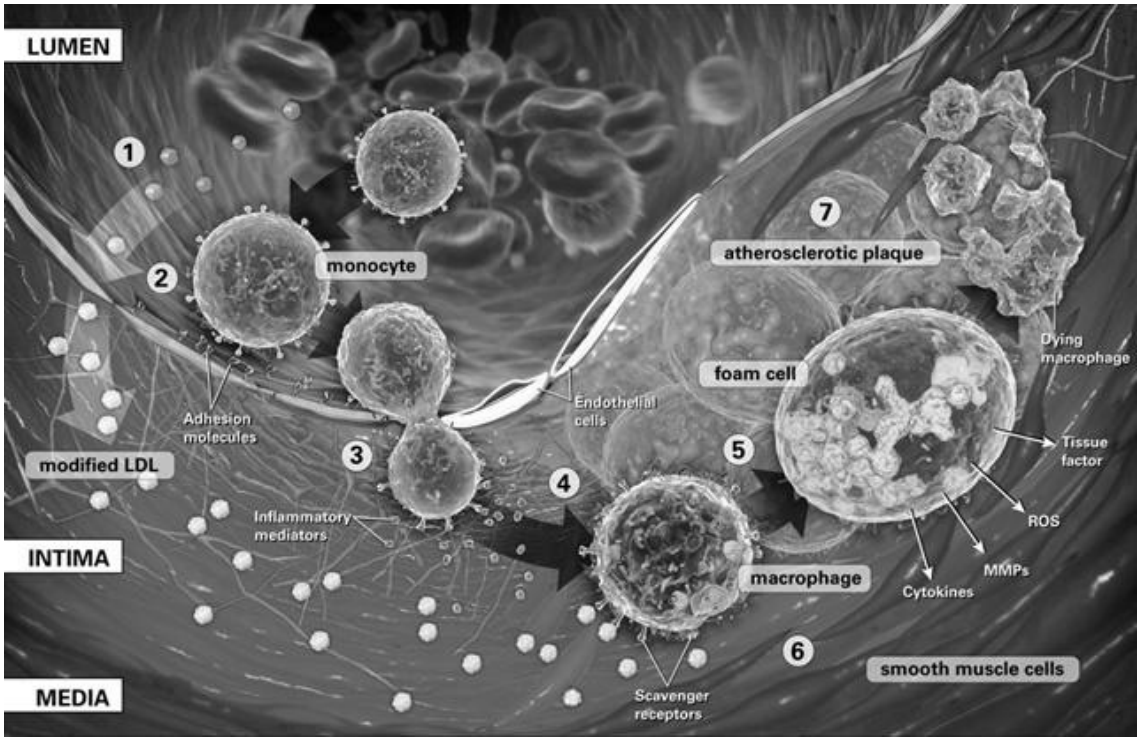

Figure 1.3. Formation of an atherosclerotic plaque (© 2010 InViVo Communications™ Inc.) Figure 1.3 shows the stages in the formation of an atherosclerotic plaque; the section below describes the stages of plaque formation; bold numbers in brackets in the section below help marry the description with the stages in the illustration.

In hypercholesterolaemia LDL enters the endothelium [**1**], here it can, among other modifications, be lightly oxidised. Lightly oxidised LDL has some pro-inflammatory properties and can contribute to the activation of endothelial cells. Activated endothelial cells have upregulated expression of cytokine inducible adhesion molecules, from the immunoglobulin gene superfamily, such as vascular cell adhesion molecule 1 (VCAM-1) and inter-cellular adhesion molecule 1 (ICAM-1). VCAM-1 on the endothelial cell surface is able to attach to cells expressing VLA4 particularly monocytes and T-cells [**2**] (138). Once attached monocytes are easily able to enter the intima via tight cell junctions, attracted by chemokine gradients of molecules such as monocyte chemo attractant protein-1 (MCP-1) [**3**]. Within the intima monocytes proliferate and differentiate into macrophages [**4**]. Macrophages in the presence of highly oxidised LDL internalise it via scavenger receptors and turn into foam cells [**5**]. Foam cells produce inflammatory cytokines, reactive oxygen species (ROS) and MMPs [**6**]. A mixture of foam cells and extracellular lipids deposited from dead foam cells form the necrotic core of the plaque [**7**]. Cytokines and growth factors produced from the macrophages and T-cells within the lesion induce smooth muscle cell migration and proliferation. Smooth muscle cells cover the foam cells and lipid laden necrotic core forming a fibrous cap along with matrix proteins, such as collagen and proteoglycans (123, 132, 137).

1.3.7 MMPs and atherosclerosis

The stability of a fibrous cap depends upon the thickness and integrity of the ECM that gives it scaffolding, strength and structure. As MMPs can degrade nearly all the constituents of the ECM, participate in inflammation and play a crucial role in tissue and vascular remodelling, it stands to reason that they are involved in atherosclerosis; numerous studies have taken place to elucidate this involvement. Just as in the case of cancer the relationship between atherosclerosis and MMPs is not straightforward. Genetic studies in MMP1 have seemingly produced conflicting results; one study finds that the more transcriptionally active 2G allele of the rs1799750 polymorphism confers a decreased risk of CHD (139), while another study finds that the same allele confers an increased risk of carotid stenosis (140). Numerous studies associate increased levels of MMP9 with increased risk or incidence of unstable angina, acute MI, and coronary artery disease (141); less consistent results were found in murine models of atherosclerosis and MMP9 deficiency, Johnson *et al*, in 2005 (142) showed that the atherosclerotic lesions in the braciocephalic arteries of knockout mice were significantly larger than those in the controls suggesting a protective role for this enzyme; in the same year Choi *et al* (143) found a reduction in intimal lesion size and volume in knockout mice compared to controls, this time suggesting a detrimental role. A summary of the experimental and clinical findings on MMPs and CAD is shown below (table 1.7; adapted from (141)).

Table 1.7. MMPs in atherosclerosis (adapted from (141))

MMP	Sources in plaque	SNPs	Mouse models; atherosclerosis phenotype	As Biomarkers for CAD
MMP-1 (collagenase 1)	Endothelial cells, SMCs, macrophage	rs1799750 (2G) associated with CAD, not with carotid stenosis; rs1144393-rs514921(A-C haplotype) increased risk of MI	hMMP1$^{+/+}$ ApoE$^{-/-}$; decreased	Higher levels found in UA, acute MI, CAD, carotid stenosis. Other studies found levels to be unchanged in CAD
MMP-8 (collagenase 2)	see next section	-	-	-
MMP-13 (collagenase 3)	Endothelial cells, SMCs, macrophage	-	MMP13$^{-/-}$ ApoE$^{-/-}$; increased collagen	-
MMP-2 (gelatinase A)	Endothelial cells, SMCs, fatty streaks, plaques	rs243864 associated with CAD	MMP2$^{-/-}$ ApoE$^{-/-}$; decreased	Higher levels found in UA, stable angina, and acute MI
MMP-9 (gelatinase B)	Leukocytes and their precursors	rs3918242 associated with CAD, peripheral arterial occlusive disease; rs78639889 (CA) carotid stenosis	MMP9$^{-/-}$ ApoE$^{-/-}$; decreased an increased	Higher levels found in UA, acute MI, CAD, carotid stenosis, peripheral artery disease (many studies)
MMP-3 (stromelysin 1)	SMCs, macrophage, lymphocytes	rs3025058 associated with carotid stenosis, reduced risk of MI	MMP3$^{-/-}$ ApoE$^{-/-}$; increased	Found decreased in symptomatic CAD, increased in first 96 hours post MI, acute coronary syndrome
MMP-10 (stromelysin 2)	Endothelial cells, macrophage	-	-	Associated with intima-media thickness
MMP-11 (stromelysin 3)	-	-	MMP11$^{-/-}$; increased intimal hyperplasia	-
MMP-7 (matrilysin 1)	Macrophage	rs11568818 associated with CAD	MMP7$^{-/-}$ ApoE$^{-/-}$; neutral	Higher levels found in CAD
MMP-14 (membrane-type1- MMP)	SMCs, macrophage	-	MMP14$^{-/-}$ bone marrow transfer to LDLR$^{-/-}$; neutral	-
MMP-12 (macrophage elastase)	Macrophage	rs2276109 associated with CAD	MMP12$^{-/-}$ ApoE$^{-/-}$; decreased, other study found neutral	Higher levels found in CAD

1.3.7.1 MMP8 in atherosclerosis

Despite being the most potent processor of native fibrillar type I collagen, the major structural component ECM in plaques (61-63, 144), MMP8 was first overlooked as a candidate in plaque rupture because it was only thought to be expressed in maturing neutrophils; the cellular composition of plaques was reported to rarely contain neutrophils (145). It has since been shown, however, that endothelial cells, smooth muscle cells and mononuclear phagocytes within atherosclerotic lesions all express MMP8 (Herman, 2001) (146). Further lines of evidence suggest a role for MMP8 in atherosclerosis; elevated serum and plasma levels of MMP8 have been associated with worst cardiovascular outcome, and the presence and severity of CAD (147-149). Levels of active MMP8 were found to be higher in rupture prone or progressive plaques, co-localising with macrophages at the shoulder region of fibrous caps (150, 151).

1.4 Aims and objectives

The overall aim of this project was to investigate effects of variations of MMP1 and MMP8 genes in the development of cancer and atherosclerosis.

Objectives:

1. To investigate effects of variation in the MMP1 gene on MMP1 promoter activity in cancer cells.

2. To conduct genetic epidemiological studies to investigate genetic variation in MMP1 and MMP8 regarding breast cancer and malignant melanoma.

3. To perform a functional characterisation of the genetic variants, including the MMP1 polymorphism rs19799750, previously associated with multiple cancers.

4. To study whether MMP8 played a role in the development of atherosclerotic lesion formation using a mouse model and to investigate the related mechanisms.

5. To conduct genetic epidemiological studies to investigate whether MMP8 polymorphisms were associated with the development and progression of atherosclerosis.

2.0 Materials and Methods

2.1 Subjects

2.1.1 Southampton Atherosclerosis Study - Southampton CAD patients

Consecutive British European CAD patients were recruited from the Wessex Cardiothoracic Unit, Southampton General Hospital. All patients had > 50% Stenosis in at least 1 epicardial coronary artery, as determined by coronary angiography. Clinical and demographic data were recorded including age, gender, weight, height, smoking habit, the presence or absence of hypertension (defined as diastolic blood pressure >95mmHg and/or systolic blood pressure >160 mmHg), the presence or absence of type 1 or type 2 diabetes (152). The clinical and demographic characteristics of the Southampton CAD patients are presented below.

Table 2.1. Demographic and clinical characteristics of the Southampton CAD patients (n=2000)

Variable	means (SD) or %
Age - year	63.5 (10.0)
Male gender	74.4%
Body mass index	27.4 (4.3)
Smoking	72.8%
Hypertension	42.4%
Diabetes	12.7%

SD, standard deviation

2.1.2 Leuven breast cancer study

Between the years 2005 and 2006, 140 patients newly diagnosed with primary operable breast cancer at the Multidisciplinary Breast Centre of the University Hospital of Leuven were recruited into the Leuven breast cancer study. Clinical and pathological data was extracted from patient files and reports. Typing of primary tumours was performed under WHO guidelines with the Elston & Ellis system used for tumour grading (153). Lymph nodes pathological examination was with haematoxylin and eosin using 3 sections per node. To reduce the chance of false negative results sentinel lymph nodes classified as negative were additionally stained with epithelial markers to detect micrometastases (48). Breast cancer staging was carried out according to the tumour-node-metastasis (TNM) system, which classifies breast cancers according to size, lymph node involvement, and metastasis (154).

2.1.3 Strangeways breast cancer case/control study

The case/control samples described below are referred to as "Set 1" by the research laboratory in possession of the study set: Cancer Research UK Department of Oncology, Strangeways Research Laboratory. Participants in both the cases and controls were of > 98% European Caucasian phenotype (155, 156).

2.1.3.1 Strangeways breast cancer cases

Strangeways breast cancer cases were drawn from the 'Studies of Epidemiology and risk Factors in Cancer Hereditary' (SEARCH) study. Recruitment took place via the

Eastern Cancer Registration and Information Centre ECRIC, Cambridge, UK. Inclusion criteria were women under the age of 55 years diagnosed with invasive breast cancer between January 1, 1991 and June 30, 1996; median age 48. Also admitted were women below the age of 70 years diagnosed from 1996 onwards; median age 48. The total number of cases available for analysis under the above criteria was 4470, the 2270 cases analysed were randomly chosen from the first 3500 women recruited. There was no significant difference in the morphology, histopathologic grade, or clinical stage of the cases analysed or not analysed (156, 157).

Strangeways controls

Strangeways controls were selected at random from participants of the Norfolk component of European Prospective Investigation of Cancer (EPIC). The controls inhabit the same geographic region as the cases and have similar median age (155, 156).

2.1.4 Bruneck study

The Bruneck Study is a prospective population-based survey on the epidemiology and aetiology of carotid atherosclerosis. 1000 healthy subjects were randomly recruited from the population of the Bruneck area in the Bolzano province, Italy in 1990. Subjects were then stratified by age and sex (125 Men and 125 women in each of the fifth to the eighth decade, n=1000). The total number of people to participate in the study was 936 with data collection complete 919 subjects. At 5 year intervals follow ups took place, in 1995 owing to death or relocation away from the area 826 were available, the second

follow up took place in the summer of 2000. Within the current study population genotyping was successful for 782 participants. Atherosclerosis was assessed, initially and in each follow up, by means of ultrasonography measuring carotid intima-media thickness (cIMT), a biomarker of carotid artery atherosclerosis. Blood samples were taken after an overnight fast and 12 hours of abstinence from smoking. Plasma levels of inflammation markers were measured by standard methods, including soluble VCAM-1 (ELISA, R&D Systems). Demographic and clinical characteristics of the subjects are shown below (158-161).

Table 2.2. Demographic and clinical characteristics of the Bruneck study subjects (n=782)

Variable	means (SD) or %
Age – year	62.8 (11.1)
Male gender	49.4%
Body mass index	25.6 (3.9)
Smoking	19.3%
Hypertension	68.1%
Diabetes	10.0%

SD, standard deviation

2.1.5 Polish breast cancer and melanoma study

Polish study cases were recruited from the International Hereditary Cancer Center, Szczecin, Poland.

2.1.5.1 Polish melanoma cases

Melanoma cases consisted of 300 consecutive individuals (175 women and 125 men) with histologically confirmed malignant melanoma of the skin (including in-situ and

invasive tumours) treated in Szczecin between 2003 and 2006; mean age at diagnosis was 55 years.

2.1.5.2 Polish breast cancer cases

Breast cancer cases consisted of 300 prospectively ascertained cases of consecutive invasive breast cancer treated in Szczecin; mean age at diagnosis was 56 years.

Polish controls

Melanoma controls were 300 healthy adults (175 women and 125 men, mean age of 55 years), who had a negative cancer family history and were sex matched and age matched (±2 years) with the patients with melanoma. The control participants were drawn from a larger study involving the residents of West Pomerania, Poland. Healthy adults had a negative family of cancer. Exclusion criteria were cancers among 1st or 2nd degree relatives.

Breast cancer controls were 300 healthy women (mean age of 56 years) who had a negative cancer family history and were age matched (±2 years) with the breast cancer cases. Other than gender, the population, inclusion and exclusion criteria were the same as the melanoma controls. Controls for melanoma and breast cancer were not necessarily mutually exclusive (162).

2.2 Genetic investigations into the role of MMPs in breast cancer, malignant melanoma and atherosclerosis

2.2.1 DNA extraction from whole blood

Genomic DNA from the different sets of subjects described above, with the exception of genomic DNA from subjects of the Leuven study, was extracted either by colleagues or collaborators (152, 158, 163-165). DNA was extracted from blood samples from the Leuven breast cancer study by a salting out method adapted from Miller et al 1988 (166). Peripheral blood samples collected into EDTA coated tubes were shipped from Belgium to Southampton on dry ice. Before DNA extraction the samples were stored at -80°C for at least 24 hours as this, anecdotally, increases the final yield. Prior to extraction samples were thawed overnight at 4°C on a rolling mixer. In a 50 ml conical tube (Falcon) 5 ml of blood was mixed with 30 ml of cold 1X erythrocyte lysis buffer (ELB) (appendix 1) for 15 minutes on ice on a rotary shaker at a slow speed for 15 minutes. The tube was then centrifuged at 500 g at 4°C for 10 minutes. The supernatant was discarded and the pellet resuspended in a further 30 ml of 1X ELB and mixed as described previously for 15 minutes. The tube was spun again at 500 g, 4°C, for 10 minutes and the supernatant discarded. The pellet was then washed into a 15 ml conical tube topped up with 1X ELB and centrifuged at 500 g, 4°C, for 10 minutes, and the supernatant discarded. The pellet was resuspended in 1-2 ml of nuclear lysis buffer (NLB) (appendix 1), followed by overnight protease K digestion at 37°C using 0.2 ml of 10% SDS and 0.5 ml of proteinase K (Sigma) solution (1 mg/ml proteinase K, 1% SDS and 2 mM Na2EDTA) in a shaker incubator. 750 µl of saturated NaCl solution was added to the overnight digest, shaken vigorously for 15 seconds, and spun at 3400 g for 15 minutes at room temperature. As much supernatant as possible (approximately 3 ml)

was transferred into a fresh 15 ml conical tube to which 2X the volume of ice cold absolute ethanol was added. The tube was inverted gently until the DNA could be seen to form a fluffy precipitate. The precipitated DNA was removed using a 200 µl pipette tip or glass rod and placed into a 1.5 ml microfuge tube containing 1 ml 70% ethanol. The microfuge tubes were pulsed on a centrifuge to pellet the DNA, the ethanol removed and the pellet allowed to air dry. 300-500 µl TE buffer was added to each pellet, depending on size, in which it dissolved over 24-48 hours. Quantification of genomic DNA was carried out by colleagues using Picogreen (167).

2.2.2 SNP selection and genotyping of the MMP1 gene

2.2.2.1 SNP selection and genotyping of the MMP1 promoter within subjects from the SAS study

In order to identify common haplotypes and the level of LD between SNPs a group of 276 unrelated individuals of British Caucasian phenotype, drawn from the SAS study, were genotyped for 7 proximal promoter SNPs of the human MMP1 gene (rs1799750, rs473509, rs498186, rs1144393, rs475007, rs514921, and rs494379). The polymorphisms had previously been identified by the PCR amplification and sequencing of the 1.9 kb *MMP1* promoter in 30 unrelated British Caucasians (24). The genotyping involved the PCR amplification of the polymorphic sequences and digestion by suitable restriction endonucleases which cleaved one of the alleles, allowing discrimination following visualisation on an agarose gel. The genotyping was performed in Southampton; the amplification primer sequences and restriction endonucleases used for each SNP can be found in appendix 2.

2.2.2.2 SNP selection for genotyping MMP1 locus within individuals from the Polish breast cancer and melanoma study

18 SNPs at the MMP1 locus were selected using the Tagger algorithm (168) which was configured to select tagging SNPs with the minimum threshold for pairwise correlation coefficients (r^2) of 0.8. The Tagger algorithm forms part the Haploview LD and haplotype analysis software available from the HapMap website. The MMP1 SNPs selected for analysis were: rs498186, rs1144393, rs475007, rs514921, rs494379, rs3213460, rs470358, rs996999, rs5031036, rs491152, rs1051121, rs11225426, rs71250626, rs2071231, rs470215, rs2071230, rs7945189, and rs1729376.

2.2.3 SNP selection for the genotyping of MMP8

2.2.3.1 in relation to breast cancer in the Leuven & Strangeways studies

The HapMap database was used to identify common SNPs (>0.05 minor allele frequency (MAF)) in the MMP8 gene region, including 3.5 kb upstream and downstream. In January 2006, when the database was queried, there were 35 SNPs that met these criteria. To cover these 35 SNPs the Tagger algorithm was configured to select tagging SNPs with the minimum threshold for pairwise correlation coefficients (r^2) of 0.8, and forced to include the promoter SNPs rs11225395 and rs1320632, previously suggested to have a functional role in MMP8 expression (50) and rs1940475 a non-synonymous exon 2 SNP. The SNPs selected for analysis were rs10895353, rs7943404, rs11225395, rs1320632, rs1940475, rs1892886, rs17099436, rs2508383, and rs1276284.

2.2.3.2 in relation to breast cancer in the Polish breast cancer study

Genotyping for the Polish breast cancer and melanoma study was subsequent to that involving the Leuven & Shanghai populations; based upon the those prior results it was decided that the rs11225395 would be a worthwhile candidate to test.

2.2.3.3 in relation to atherosclerosis

MMP8 SNPs to genotype in relation to atherosclerosis were identified by the re-sequencing described below and tagging SNPs selected from the HapMap database to capture ($r2 \geq 0.8$) common SNPs (MAF ≥ 0.05) in the introns, 5' upstream sequence and 3'-untranslated region. The SNPs are detailed in the results section Table 3.10.

2.2.4 MMP8 resequencing

All 10 exons of MMP8 were amplified from the genomic DNA of 30 unrelated individuals of British Caucasian phenotype, drawn from the SAS study, using Touchdown PCR (TDPCR). The sequencing of 30 unrelated individuals gives the study a power of more than 90% to detect polymorphisms with a MAF >5% (169). The primers used to amplify the MMP8 exons were designed using the online primer design program 'Primer3'(170) using sequence information available from the 'Ensembl' genome browser (www.ensembl.org) build 39 (171). The Primer sequences can be found in appendix 3.

2.2.4.1 Touchdown PCR

The polymerase chain reaction (PCR) is one of the most widely used and fundamental techniques used in biological research. The technique exponentially amplifies DNA from a given starting template. The enzyme responsible for this reaction a thermostable DNA polymerase, isolated from the bacterium *Thermus aquaticus*, is commonly referred to as Taq polymerase or even Taq. The cyclic process of DNA amplification takes place in 3 main stages; denaturation of double stranded DNA, annealing of primer oligonucleotides to the single stranded DNA, and extension of a complementary strand of DNA, from the double stranded start position provided by the annealed primers, along the single strand. Complete denaturation of double stranded DNA typically takes place around 95ºC; the temperature for the annealing of primers is dependent upon their size and base composition but is commonly around 60ºC. Taq polymerase's optimum temperature for extension is 72ºC. This amplification cycle doubles the amount of DNA each time it occurs. Targeted amplification is achieved with the use of specific primers; since both strands can be used as templates, the primers can be chosen to flank the region of interest. The thermostability of Taq removes the need for replacement of enzyme for each cycle, a step previously required when the DNA polymerases used would be inactivated by the high temperatures needed for DNA denaturation; this has enabled PCR to become a routine automated process carried out on thermal cyclers (172). TD PCR is a modification of PCR designed to reduce mis-priming and the generation of non-specific products, without the need for time consuming empirical optimisation of all conditions. The method relies the fact that higher temperatures increase the stringency of the reaction by favouring correct base hybridisation of primers over mis-matched base priming; additionally sub - melting point (Tm) annealing temperatures that occur towards the end of the touchdown stage help to

increase the overall yield of the reaction, after sufficient correct template has been created by the previous higher annealing steps, when buffer conditions may be less than optimal. Tm is defined as the temperature at which one half of a DNA duplex molecule is dissociated into single strands. TD PCR typically starts with an annealing temperature that is several degrees higher than the expected Tm of the primers used in the reaction; the touchdown stage ends with the annealing temperature slightly lower than the expected Tm of the reaction primers. Subsequent to the touchdown stage there are 20 - 30 cycles of denaturation, annealing and extension with the annealing temperature 2 - 5ºC lower than the calculated Tm. All 10 amplicons of MMP8 exons including intron junctions) were amplified with the following TD PCR protocol template.

Table 2.3. Touchdown PCR cycling conditions

Step	Temperature /Condition	Time/Additional cycles
01	95°C	10 min
02	95°C	30 sec
03	65 - 68°C	30 sec (-1°C/cycle)
04	72°C	1 minute
05	Goto step 02	X 9
06	95°C	30 sec
07	55 - 58°C	30 sec
08	72°C	1 minute
09	Goto step 06	X 29
10	72°C	10 minutes
11	End	

In a 30 µl reaction 3 µl of 10X Amplitaq Gold PCR buffer (Applied Biosystems) was added to 21.15 – 20.55 µl of H_2O followed by 1.2 µl of dNTPs (5 mM), 2.5 - 3 µl of $MgCl_2$ solution (25 mM), 0.1 µl of each primer (10 µM) and 0.05 µl of Amplitaq Gold DNA Polymerase (5.0 U/µl, Applied Biosystems). 2 µl of genomic DNA template (7

ng/µl) was added to the relevant reaction. PCR was performed on an on an MJ-Tetrad PTC-225 thermal cycler.

2.2.4.1.1 PCR optimisation

The optimal reaction conditions for PCR primer pairs were typically determined by testing a range of annealing temperatures starting 2°C below the manufacturer stated Tm up to 2°C above, in conjunction with four different $MgCl_2$ concentrations 1 mM – 3 mM.

2.2.4.2 Agarose Gel Electrophoresis

This technique allows the characterisation and visualisation nucleic acids, separating DNA or RNA fragments. It is an important step in the purification of specific DNA sequences. Agarose gel provides a matrix of cross-linked molecules; enabling DNA fragments to be separated by their size, in the presence of an electric field. Nucleic acids are charged molecules; most DNA fragments will have a comparable size charge ratio. When an electric field is applied to a gel DNA will migrate towards the positive terminal (anode). The DNA within the gel is visualised with aid of dyes that fluoresce under ultra-violet light; initially in this project the dye used was ethidium bromide (EtBr) which intercalates between bases, the DNA-EtBr complex providing a strong clear band. Later on in the project the dye used was 'Gel Red' (Biotum) which works in a similar way to EtBr but is less toxic. Typically in this project 1% or 2% w/v agarose gels were used. Lower percentage gels (less cross-links) give better resolution between

larger size fragments; higher percentage gels (denser matrix) give better resolution in between small size fragments.

Separation of DNA on a 1% agarose gel was as follows:

1 g of agarose was added to 100 ml of 1x TAE or 1x TBE buffer (appendix 1) in a 250 ml Pyrex glass conical flask; This mix was then heated to boiling and allowed to cool for 2 minutes while being stirred with a magnetic stirrer. 6 µl of Gel Red nucleic acid dye was then added and the flask was allowed to cool until it was comfortable to hold. The gel was then poured into a rectangular Perspex mould and allowed to set with a rigid comb in place to create sample-loading wells. The gel is submerged in 1x TAE/TBE buffer solution, in a gel electrophoresis tank, that acts as an electrolyte between the positive and negative electrodes. DNA samples were mixed with DNA loading buffer, to give them a higher density than the TAE or TBE buffer and provide a visual reference of the progression of the electrophoresis, and then loaded into the wells. Along with the samples a DNA weight marker was used, this consists of fragments of known size that provide a reference against the PCR products. The gel was run under constant voltage at 110 V for 45 minutes. Samples were visualised on a dual intensity trans-illuminator under UV-light.

2.2.4.3 Sequencing

Sequencing of MMP8 amplicons was performed externally by Kbiosciences using an ABI 3100 DNA Sequencer. Analysis of the sequencing results, sent from Kbiosciences, and SNP identification was performed using the Seqman tool, an application which

forms part of the Lasergene sequence analysis software (DNASTAR). SNPs were detected either visually as heterozygotes, showing clear 'peaks within peaks' (Fig 3.7), or as homozygotes differing from the publically available consensus sequence.

2.2.5 Genotyping of MMP8 in relation to breast cancer

2.2.5.1 Genotyping of MMP8 within the Leuven study population and the Strangeways breast cancer case/control study

Breast cancer patients from the Leuven study and patients and controls from the Strangeways breast cancer case/control study were genotyped for the 9 polymorphisms mentioned in section 2.2.3.1 using the Taqman SNP genotyping system (Applied Biosystems) based upon the 5' nuclease assay. The Applied Biosystems assays used for each SNP are listed in the table below.

Table 2.4. List of MMP8 SNPs genotyped in the Leuven Breast Cancer Study population and Strangeways breast cancer case/control study with corresponding ABI assay #

SNP	ABI assay #
rs10895353	C__27134501_10
rs7943404	C__29361216_10
rs11225395	C___1366493_10
rs1320632	C___7492587_10
rs1940475	C__11484594_1_
rs1892886	C__11484595_10
rs17099436	C__34384563_10
rs2508383	C___7492589_10
rs1276284	C___7492595_10

The allelic discrimination of the Taqman 5' nuclease assay relies on the amplification and 5' nuclease activities of the enzyme Taq Polymerase. In the assay forward and

reverse primers, adjacent to the region of interest, are combined with 2 short allele specific oligonucleotide probes each labelled with either the Applied Biosystems VIC (VIC) or 6-carboxy-fluorescein (FAM) fluorescent dyes, along with a quencher dye. For unbound probe the proximity of the quencher molecule drastically reduces the fluorescent signal of the fluorophores. During the polymerisation step of PCR Taq Polymerase, on encountering specifically hybridised probe, cleaves the probe with its 5' nuclease activity; the fluorophores produce a readily quantifiable signal subsequent to their release from the proximity of the quencher molecule (173).

The procedure for the genotyping of the samples is as follows; 5 ng genomic DNA samples were added to 384 well optical reaction PCR plates. The DNA was allowed to air dry over 24-48 hours. A master mix of the reaction components was prepared with sufficient quantity for the total number of samples plus 10% to account for pipetting errors. The total volume of each reaction was 5 µl; each reaction contained 2.5 µl 2X Taqman Universal Master Mix, 0.25 µl 20X SNP Genotyping assay Mix, and 2.25 µl water. 5 µl of the reaction mix was pipetted into each well, the plate sealed with an optical adhesive cover, and spun down in a centrifuge at 2000 g for 1 minute. PCR was performed on an MJ-Tetrad PTC-225 thermal cycler; the cycling conditions were 95°C initiation for 10 min, followed by 40 cycles of denaturation at 92°C for 15 seconds and annealing/extension at 60°C for 1 minute. Plates were read using the Applied Biosystems 7900HT sequence detection system. The results were then analysed on Applied Biosystems SDS analysis software V2.1.

2.2.5.2 Genotyping of MMP1 and MMP8 within Polish cancer patients and controls

As above, the genotyping of MMP1 and MMP8 within Polish cancer patients and controls used 'off the shelf' Taqman assays (Applied Biosystems). The methodology remained the same with the exception of the volumes used. A single reaction consisted of : 5 ng dried genomic DNA, 1 µl of Taqman assay universal master mix, 0.05 µl of SNP genotyping assay mix and 0.95 µl of sterile, deionised water.

2.2.6 Genotyping of MMP8 in relation to atherosclerosis

1000 CAD patients from Southampton General Hospital were genotyped for MMP8 SNPs rs1276284, rs11225395, and rs1940475 using the Taqman SNP genotyping system (Applied Biosystems), and the respective assays C___7492595_10, C___1366493_10, and C__11484594_1_; the methodology was as described previously.

2.2.6.1 Genotyping using KASPar

The 1000 CAD patients from Southampton General Hospital were also genotyped for the other SNPs selected (rs10895353, rs7943404, rs1320632, rs2155052, rs17099450, rs3765620, rs12803000, rs1892886, rs17099436, rs3740938, rs2508383, rs1276282, rs1291327) by KBiosciences using the KASPar system. KASPar is a proprietary genotyping system developed by KBioscience LTD. The system is a competitive PCR assay that utilises the specificity of Taq polymerase with the detection of FAM™ and VIC™ labelled oligos, non-fluorescent quencher molecules and passive reference dye

ROX™. The system requires 2 allele specific probes, 1 for each allele, combined with a common primer. These unlabelled oligos can either be designed by KBioscience or with the use the design program 'Primer Picker' available at www.kbioscience.co.uk/primerpicker. The non-synonymous SNP rs1940475 was associated with the extent of coronary atherosclerosis and remained so after correction for multiple SNP testing. A further 1000 CAD patients from Southampton general hospital were then genotyped for this SNP. Subjects from the prospective population-based Bruneck Study were also genotyped for the rs1940475 SNP.

2.2.7 Determination of linkage disequilibrium between MMP1 promoter polymorphisms and identification of common haplotypes

The LD between the 7 MMP1 promoter SNPs and the frequencies of common haplotypes was determined from the genotype data using the Haploview program, which implements an accelerated expectation-maximisation (EM) algorithm (174, 175). Colleagues concurrently utilised the THESIAS and PHASE_V2.1 programs to the same ends. THESIAS uses the Stochastic-EM algorithm (176) and PHASE uses a Bayesian method (177, 178). The Haploview program may be downloaded from the following web address: http://www.broadinstitute.org/haploview/haploview-downloads

2.2.8 Acquisition and presentation of MMP1 promoter genetic recombination rates

Data and graphical representations pertaining to the recombination rates in and around the MMP1 gene were obtained by colleagues from the HapMap database: http://hapmap.ncbi.nlm.nih.gov.

2.2.9 In silico analysis of transcription factor binding sites lost or gained in the MMP1 promoter, as caused by SNPs

It was assessed whether alleles of the SNPs studied in the MMP1 promoter caused a loss or gain of putative transcription factor binding sites using the SNPInspector program; SNPInspector is a variant of the Genomatix program MATInspector (179).

2.2.10 Statistical analysis of MMP8 gene variation in relation to cancer

2.2.10.1 Statistical analysis of MMP8 gene variation and breast cancer prognosis

Statistical analysis involving the Leuven breast cancer study was performed by colleagues; the gene counting method was used to calculate allele and genotype frequencies. Association between clinical and pathological characteristics and genotypes or alleles was tested by $\chi 2$ analysis. The THESIAS program, which utilises a stochastic EM (Expectation-Maximisation) algorithm (176) was used to determine the SNP pair-wise linkage disequilibrium coefficient, haplotype frequencies, and any haplotypic effects on lymph node metastasis.

2.2.10.2 Statistical analysis of MMP8 gene variation and breast cancer in the Strangeways study

Genotypic statistical analysis involving the Strangeways breast cancer case/control study was performed by collaborators at the Strangeways Research Laboratory (156);

the gene counting method was used to calculate genotype frequencies. Allelic association was calculated using PLINK V1.07 (180).

2.2.10.3 Statistical analysis of MMP8 gene variation in the Polish breast cancer and melanoma study

Statistical analysis involving the Polish patients and controls was performed by collaborators in Poland. χ^2 analysis was used to assess whether allelic and genotypic frequencies differed from those expected under Hardy–Weinberg equilibrium (HWE). Potential haplotype effects of MMP1 variation on cancer were assessed using the THESIAS program. Since the Polish study involved matched case-controls, conditional logistic regression was used calculate disease risk associated with particular genotypes or alleles. Multiple testing was taken into account by the use of all other SNPs as covariates when querying each SNP.

2.2.10.4 Meta-analysis of data from the analyses of the MMP8 SNP rs11225395 in the Strangeways and Polish breast cancer studies

The meta-analysis function of PLINK V1.07 (180) was used to assess the combined effect sizes of any association between the rs11225395 SNP and breast cancer in the Strangeways and Polish case/control studies. The meta-analysis function of PLINK tests both random and fixed effect models, heterogeneity is quantified using both Cochran's Q test (181) and the I^2 heterogeneity index (182).

2.2.11 Statistical analysis of genotyping data for MMP8 SNPs in relation to atherosclerosis

Statistical analysis of genotyping data for MMP8 SNPs tested in CAD patients from Southampton and members of the Bruneck study cohort was performed by colleagues. Logistical and linear regression analyses were performed to uncover any significant relationship between the number of coronary arteries with >50% stenosis and MMP8 SNPs in Southampton CAD patients and whether there was a relationship between the progression of carotid atherosclerosis in the Bruneck Study cohort and the rs1940475 SNP. ANOVA was used to test a difference in plasma soluble VCAM-1 level between rs1940475 genotypes in the Bruneck study cohort. To account for multiple testing, 16 SNPs in the Southampton CAD patients, the SNPSpD method (183, 184) was used, giving a significance threshold of 0.005; this method was not required for the interpretation of rs1940475 data in the Bruneck study as only 1 SNP was tested, therefore the significance threshold of 0.05 was used.

2.3 Functional investigations of MMP genes in cancer and atherosclerosis

2.3.1 Generation of MMP1 promoter haplotype luciferase reporter gene constructs

All MMP1 promoter plasmid constructs were generated previously (24). For each of the 10 commonest haplotypes, identified by the Haploview, THESIAS, and PHASE programs, a 1.9 kb promoter sequence was amplified by PCR, using the genomic DNA of individuals carrying the specific haplotype as a template. The promoter sequences were then cloned into the pCR-Blunt II-TOPO vector (Invitrogen, San Diego, CA) and then subcloned via the multiple cloning site upstream of the firefly luciferase reporter gene in the pGL3-basic vector (Promega, Madison, WI). All constructs were then sequenced to verify correct orientation and absence of misincorporations.

2.3.2 Cloning of the MMP8 promoter

For each of the two alleles of the rs11225395 SNP, -799 bp relative to the transcriptional start site, in the MMP8 gene promoter, a 967 bp DNA fragment, from positions -872 to +96, was generated by PCR from the genomic DNA of individuals homozygous for each allele. The PCR primers used to clone the MMP8 promoter fragment were:

MMP8_prom_F: 5’ CTGTTGAAGGCCTAGAGCTGCTGCTCC 3’

MMP8_prom_R: 5’ GATCTTCTCTTCAAACTCTACC 3’

In a 50 µl reaction 5 µl of 10X Pfx amplification buffer was added to 35.5 µl of H_2O followed by 3 µl of dNTPs (5 mM), 1 µl of $MgSO_4$ solution (50 mM), 1.5 µl of each primer (10 µM) and 0.5 µl of Platinum® Pfx DNA Polymerase (2.5 U/µl, Invitrogen). 2 µl of genomic DNA template (7 ng/µl) was added to the relevant reaction. The cycling conditions on a MJ Tetrad PTC – 225 thermal cycler were 94°C for 2 minutes, then 25 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute. The PCR products were checked on a 1.5% TBE agarose gel and purified using a QIAquick gel extraction kit (Qiagen).

The purified products were then cloned into the pCR-Blunt II-TOPO vector (Invitrogen) using the Zero Blunt® TOPO® PCR cloning kit (Invitrogen). To 4 µl of PCR product 1 µl salt solution (1.2M NaCl, 0.06M MgCl2), and 1 µl of TOPO vector (10 ng/µl) were added; this reaction was left to incubate at room temperature for 5 minutes before being placed on ice. 2 µl of the cloning reaction was added into a vial of One Shot® chemically competent *E.coli* and mixed and incubated on ice for 10 minutes. The cells were then heat-shocked for 30 seconds at 42°C and transferred onto ice. 250 µl of room temperature S.O.C. medium (Invitrogen; cat#15544-034) added to each tube which were then capped and incubated for 1 hour at 37°C at 200 rpm in a shaking incubator. 50 µl from each transformation was spread on a pre-warmed (50 µg/ml kanamycin) selective plate and incubated overnight at 37°C.

Transformants were picked the following morning and transferred onto a numbered grid lined reference Luria Bertani agar (LB) plate and incubated 37°C for several hours. Selected colonies were grown up overnight in 3 ml of LB Broth (50 µg/ml kanamycin) in 15 polypropylene ml tubes at 37°C at 200 rpm in a shaking incubator. Plasmids from

the transformed colonies were purified using the QIAprep miniprep kit (Qiagen). Clones were verified against misincorporation and mis-orientation by sequence analysis.

2.3.2.1 Subcloning of MMP8 gene promoter inserts into the pGL3-basic luciferase reporter vector.

The MMP8 gene promoter inserts were directionally subcloned from the pCR-Blunt II-TOPO vector into the pGL3-basic luciferase reporter vector (Promega) by restriction with restriction endonucleases KpnI and XhoI (New England Biolabs) and T4 DNA ligase (Roche). 1 µg of MMP8-pCR-Blunt II-TOPO was added to 10U of KpnI (10U/µl), 10U of XhoI (10U/µl), 3 µl 10X NEBuffer 1, 0.3 µl of BSA (Bovine serum albumin, 10 mg/ml), H_2O to make up to 30 µl and incubated for 3 hours at 37°C. The pGL3-basic vector was linearised using the same enzymes and conditions. Both digests were run and separated on a 1.5% gel by agarose gel electrophoresis, the relevant inserts and vector purified with the QIAquick gel extraction kit (Qiagen). Ligation was performed using a 1:3 molar ratio of vector DNA to insert DNA in 1X T4 DNA ligase buffer with 1 unit of T4 DNA ligase in a 10 µl reaction incubated at 4°C for 16 hours, halted at 65°C for 15 minutes. 2 µl of the ligation reactions were transformed in the same manner described above, with the exception that the LB selective plates contained 100 µg/ml of ampicillin. Transformants were picked the following morning and transferred onto a numbered grid lined reference LB plate and incubated 37°C for several hours. Selected colonies were grown up overnight in 3 ml of LB Broth (100 µg/ml ampicillin) in 15 polypropylene ml tubes at 37°C at 200 rpm in a shaking incubator. Plasmids from the transformed colonies were purified using the QIAprep miniprep kit (Qiagen). Plasmids were screened for correct sized inserts by digestion

with restriction endonucleases KpnI and XhoI. Colonies on the reference plate that had been shown to contain the correct sized insert were grown up in 50 ml of LB broth (100 µg/ml ampicillin) in 250 ml conical flasks overnight at 37°C at 150 rpm in a shaking incubator. Plasmids were then purified using an EndoFree plasmid maxi kit (Qiagen). Clones were then checked for misincorporation by sequence analysis.

2.3.3 Cloning of the murine VCAM-1 promoter

The VCAM-1 promoter sequence (263bp) was amplified from genomic murine DNA with primers that also introduced restriction endonuclease recognition sites. The sequences were incorporated at the 5' ends of the forward and reverse primers with an additional 4 bases upstream of the respective restriction sequence to aid digestion of the amplicon creating the correct 'sticky ends' for ligation into an appropriate vector. The forward primer complementary for the 5' end of the VCAM-1 promoter sequence introduced a Kpn1/Acc65I (shown in red) restriction site. The reverse primer introduced a BglII (shown in red) recognition site. The primer sequences are shown below.

mVCAM1prom_Kpn1_F	5' GTTCGGTACCTCTTTTCAGGAGAGATAGCCCTTTC 3'
mVCAM1prom_BglII_R	5' GTTGAGATCTGCCGGGCTGGTGTGAGTGA3' +2tss

PCR products were checked for correct size (283 bp) on a gel, once the correct size was verified the products were co-digested with Acc65I and BglII (New England Biolabs) ligated with a similarly digested and linearised pGL3-Promoter (Promega) plasmid. Ligation, transformation and checking by sequencing analysis were performed as shown in section 2.3.2.1.

2.3.3.1 Ablation of NF-kB transcription site in murine VCAM-1 promoter

The promoter sequence of human VCAM-1 has been shown to contain a functional NF-kB element (185). This site is also found in murine VCAM-1, which shows a high degree of homology, with human VCAM-1, in both the gene and promoter sequences and the translated amino acid sequence (186). In order to ablate the NF-kB element within the murine VCAM-1 promoter in vitro mutagenesis was used, based upon the method demonstrated by Scott *et al* in 2002 (187). Complementary forward and reverse oligos were designed similar to those used to ablate the. NF-kB element in the human sequence by Neish *et al* in 1992 creating non-complementary transversion mutants, i.e. G changed to T, C changed to T and *vice versa* (185). Underlined nucleotides have been changed.

VCAM1PROMMUT_F	5' CTGGGTTTCCCCTTGAA<u>TTT</u>ATTT<u>AAA</u>TCCGCCTC 3'
VCAM1PROMMUT_R	5' GAGGCGGA<u>TTT</u>AAAT<u>AAA</u>TTCAAGGGGAAACCCAG 3'

In a 50 µl reaction 75 ng of the plasmid template was added to 125 ng of each oligonucleotide along with 3.75U *PfuTurbo*™ DNA polymerase (Stratagene, La Jolla, CA, USA) and buffer, and 300 µM dNTPs. Cycling was performed on an MJ Tetrad under the following conditions:

Step	Temperature /Condition	Time/Additional cycles
01	95°C	2 min
02	95°C	40 sec
03	58°C	50 sec
04	68°C	1.5 min/kb
05	Goto step 02	X15
06	End	

A 10 µl aliquot from the mutagenesis PCR is run on a 1% Agarose gel to check size of the amplicon. The correct sized amplicon was digested for 3 hours at 37°C with 30U of the restriction enzyme DpnI. DpnI will only cleave methylated or hemimethylated DNA at the sequence G^{m6}ATC. Template/Parental DNA isolated from most strains of *E.coli is* Dam methylated (188). This step removed the non-mutated template DNA and increased the efficiency of mutagenesis. The clean up used the Promega SV kit, followed by transformation into xl-gold, and verification by sequencing.

2.3.4 Cell culture

All cell culture was performed in a class II microbiological safety cabinet. The table below lists the cells lines used, a description of the cell line, source of the cells, and the gene promoters investigated.

Table 2.5. Cell lines used in this study

Cell Line	Description	Source	Gene promoter investigated
A2058	Human Caucasian metastatic melanoma	ECACC*	MMP1/MMP8
A375	Human malignant melanoma	ECACC*	MMP1
MCF7	Human Caucasian breast adenocarcinoma	ECACC*	MMP1
MDA-MB-231	Human Caucasian breast adenocarcinoma	ECACC*	MMP1/MMP8
A549	Human Caucasian lung carcinoma	ECACC*	MMP1
H69	Human Caucasian lung small cell carcinoma	ECACC*	MMP1
HT-29	Human Caucasian colon adenocarcinoma	Dr Simon Joel**	MMP1
SW-620	Human Caucasian colon adenocarcinoma	Dr Simon Joel**	MMP1
C166	Mouse endothelial cell line	ATCC***	VCAM-1
bEnd.3	Mouse endothelial cell line	ATCC***	VCAM-1

* European Collection of Cell Cultures
** Kindly donated by Dr Simon Joel, Institute of Cancer, Barts and The London School of Medicine, London, United Kingdom.
*** American Type Culture Collection

The propagation, media and culture conditions for each of the above cancer cell lines were as follows:

A2058 – melanoma cell line

A2058 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, supplemented with 4 mmol/l of L-glutamine, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

A375 – melanoma cell line

A375 cells were cultured in DMEM containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, supplemented with 4 mmol/l of L-glutamine, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

MCF7 – breast cancer cell line

MCF7 cells were cultured in Eagle's minimal essential medium (EMEM) with Earle's balanced salt solution supplemented with 2 mmol/l of L-glutamine, 1.0 mmol/l of sodium pyruvate, 0.1 mmol/l of nonessential amino acids, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

MDA-MB-231 – breast cancer cell line

MDA-MB-231 cells were cultured in Leibovitz's L15 medium supplemented with 2 mmol/l of L-glutamine, 1.0 mmol/l of sodium pyruvate, 0.1 mmol/L of nonessential amino acids, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 100% air.

A549 – lung cancer cell line

A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, supplemented with 4 mmol/l of L-glutamine, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

H69 – lung cancer cell line

H69 cells were cultured in RPMI 1640 with 2 mmol/l of L-glutamine adjusted to contain 1.5 g/l of sodium bicarbonate, 4.5 g/l of glucose, 10 mmol/l of HEPES,

1.0 mmol/l of sodium pyruvate, 10% foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

HT-29 – colorectal cancer cell line

HT-29 cells were cultured in RPMI 1640 with 2 mmol/l of L-glutamine adjusted to contain 1.5 g/l of sodium bicarbonate, 4.5 g/l of glucose, 10 mmol/l of HEPES, 1.0 mmol/l of sodium pyruvate, 10% foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

SW-620 – colorectal cell line

SW-620 cells were cultured in DMEM containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, supplemented with 4 mmol/l of L-glutamine, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

C166 – mouse endothelial cell line

C166 cells were cultured in DMEM containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, supplemented with 4 mmol/l of L-glutamine, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

bEnd.3 – mouse endothelial cell line

bEnd.3 cells (189) were cultured in DMEM containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, supplemented with 4 mmol/l of L-glutamine, 10% heat-inactivated foetal bovine serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin; the cells were incubated at 37°C in 5% CO_2.

2.3.4.1 Procedure for passage of adherent cell lines

Cells were passaged on reaching 70-80% confluency. The culture medium was removed from over the attached monolayer using a sterile serological pipette, and discarded into 1% Virkon (DuPont) solution. The monolayer was then washed with 1 to 2 ml of trypsin-EDTA; the wash solution discarded into 1% Virkon. 3 ml of the trypsin-EDTA solution was added to the flask and which was placed back into an incubator at 37ºC for approximately 5 minutes. Progress of the detachment can be checked with an inverted phase contrast microscope. While the cells were detaching 10 ml of pre-warmed fresh medium was added to the required number of new T75 flasks. Once the cells were detached 6 ml of fresh medium was added to the flask containing the cell and trypsin-EDTA solution, to inactivate the trypsin-EDTA. 3ml of the medium containing the cells was then added to the requisite flasks, achieving a 1:3 split ratio, the flask sealed and placed into the 37°C incubator. Alternatively after cells were detached, 3 ml of fresh medium was added a 0.5 ml aliquot of the cell suspension taken and added to the automated Vi-CELL XR cell viability and cell counter (Beckman Coulter). The cell viability and density values obtained were used to dilute (with fresh medium) the cell suspension to the required seeding density for seeding in T75 tissue culture flasks, 6 well tissue culture plates or 24 well tissue culture plates as required.

2.3.5 Transient transfection to investigate the effects of MMP1 promoter haplotypes in cancer cells

Transfection, in this case is the introduction of foreign DNA into human or animal cells. In transient transfection, the foreign DNA does not enter the host cell's genome and is therefore lost during subsequent cell division and mitosis. Each of the cell lines (A2058,

A375, MCF7, MDA-MB-231, A549, H69, HT-29, and SW-620) were transiently transfected with each of the haplotype reporter constructs described above, an empty pGL3-basic vector, and the pGL3-control vector; the pGL3-control vector contains the SV40 promoter upstream of the luciferase gene which induces high expression of the luciferase in mammalian cells. In all experiments the pRL-TK plasmid was co-transfected, a vector which expresses *renilla* luciferase, to serve as an internal reference for transfection efficiency. Cell lines A2058, A375, MCF7, A549, HT-29, and SW-620 were transfected using the Fugene 6 (Roche) transfection reagent. Cell lines MDA-MB-231 and H9 proving to have poor transfection efficiency with Fugene 6 were transfected with Effectene (Qiagen).

2.3.5.1 Transient transfection with Fugene 6 transfection reagent

The Fugene 6 transfection reagent is what is known as a "cationic amphiphile" due to its ability to coat DNA with positively charged lipid micelles (190), this fugene/DNA complex has the ability to disrupt and pass through eukaryotic cell membranes, thereby delivering the DNA into cells and achieving transfection (191). The procedure used with the Fugene 6 transfection reagent, adapted from the official protocol, is as follows:

Working stock DNA was made to a concentration of 50ng/µl containing 19 parts luciferase reporter construct (pGL3-MMP1_promoter_haplotype or pGL3-basic or pGL3-control) to 1 part *renilla* luciferase vector (pRL-TK). The optimal renilla/vector ratio, (20:1) ratio, Fugene 6/DNA ratio, and cell seeding densities were derived experimentally.

On the day prior to the transfection experiment cells were seeded in 0.5ml of media per well in a 24 well plate at the required cell density 6.0 x 10^5 cells/ml. The following day cell confluency was checked using a phase contrast microscope; transfection experiments were only performed in wells averaging 70-80% confluency. For transfection of the cancer cell lines a 4:1 (Fugene 6(µl):DNA(µg)) transfection mix ratio was used i.e. 0.8 µl transfection reagent and 200 ng of DNA. To make up the transfection mix 15.2 µl of room temperature serum-free media was added to a sterile 1.5 ml tube, 0.8 µl of Fugene 6 was added directly into the media without touching the sides of the tube followed by 4 µl of DNA (50 ng/µl); for transfecting more than 1 well the above amounts are multiplied accordingly (n +1). The tube containing the transfection mix was tapped gently, to mix, and incubated at room temperature for 15 minutes. After the incubation 20 µl was added dropwise to each well, swirling to allow even distribution. The plate was then returned to the 37°C incubator.

2.3.5.2 Transient transfection with Effectene transfection reagent

Effectene is a non-liposomal lipid transfection reagent; details of its DNA delivery mechanism are commercially sensitive and not publicly available, although as a cationic lipid (192) it may function in a similar manner to Fugene6. The procedure used with the Effectene transfection reagent, adapted from the official protocol, is as follows:

Working stock DNA was made to a concentration of 50 ng/µl containing 19 parts luciferase reporter construct (pGL3-MMP1_promoter_haplotype or pGL3-basic or pGL3-control) to 1 part *renilla* luciferase vector (pRL-TK).

On the day prior to the transfection experiment cells were seeded in 0.5ml of media per well in a 24 well plate at the required cell density 6.0 x 10^5 cells/ml. The following day cell confluency was checked using a phase contrast microscope; transfection experiments were only performed in wells averaging 70-80% confluency. To make up the transfection mix 54.4 µl of room temperature Buffer EC was added to a sterile 1.5 ml tube. 200 ng of DNA was then added to the tube, followed by 1.6 µl of Enhancer; the mixture was then vortexed and incubated at room temperature for 3 minutes. 5 µl of Effectene transfection reagent was then added and the contents of the tube mixed by pipetting up and down 5 times. The DNA-transfection complex was then incubated at room temperature for a further 10 minutes. During the incubation period the medium in the wells was aspirated, the cells were washed twice with 1 ml sterile PBS, and 0.5 ml fresh (pre-warmed) medium was added to each well. 350 µl of medium was then added to the DNA-transfection complex, and pipetted up and down twice. 415 µl of the transfection mix was added dropwise to each well, while swirling to allow even distribution. The plate was then returned to the 37°C incubator.

2.3.6 Transient transfection for the purpose of investigating the effects of MMP8 promoter SNPs in the MDA-MB-231 and A2058 cancer lines

Transient transfection used to investigate the MMP8 promoter was as that used for investigating MMP1 promoter haplotypes, with the substitution of the MMP8 promoter luciferase reporter constructs for the MMP1 haplotype promoter luciferase reporter constructs. MDA-MB-231 cells were transfected using Effectene and A2058 cells were transfected using Fugene 6.

2.3.7 Transient transfection used to measure the effect of the cleavage products of angiotensin I by MMP8 on VCAM-1 promoter activity

Transient transfection of C166 and bEnd.3 cells was used to measure the effect of the cleavage products of angiotensin I by MMP8 on VCAM-1 promoter activity. The transfection reagent in both cases was Fugene 6. The transfection mix ratio 3:2 (Fugene 6(µl):DNA(µg)) was found to be optimal for both cell lines; Plasmid amounts were optimised at 400 ng of the vector (either pGL3-VCAM-1, or pGL3-VCAM-1_Mut or pGL3-basic or pGL3-control) to 10 ng *renilla* luciferase vector (pRL-TK)). All other parameters were as in section 2.3.5.1.

2.3.7.1 Treatment of cells transfected with the VCAM-1 promoter

All treatments were carried out 18 hours post transfection and 6 hours pre-lysis. The medium in the wells was aspirated, the cells were washed twice with 1 ml sterile PBS, and 0.5 ml fresh (pre-warmed) medium was added to each well. The treatments (angiotensin I, MMP8, MMP8 and angiotensin I, or no treatment) were prepared as in section 2.4.1, with the relevant components added or removed. 500 µl of medium (containing the respective treatments) was added dropwise to each well, while swirling to allow even distribution. The plate was then returned to the 37°C incubator. The concentrations of MMP8 and angiotensin I were 52 ng/ml and 130 ng/ml respectively.

2.3.8 Dual-luciferase assay

The Dual-luciferase assay (Promega), used in conjunction with pGL3 luciferase vectors (Promega) relies on the expression and measurement of 2 distinct luciferases, firefly luciferase (*Photinus pyralis*) and sea pansy luciferase (*Renilla reniformis*). The firefly

luciferase serves as a readily detectable surrogate for the genes under the control of the promoters being tested by this method.

Kit components:

Luciferase Assay Buffer II

Luciferase Assay Substrate (Lyophilized Product)

Stop & Glo® Buffer

Stop & Glo® Substrate (50X)

Passive Lysis Buffer (5X)

In each experiment, all samples and controls were in triplicate (3 wells each).Dual-luciferase assays were performed 18 hours post transfection. 100 µl luciferase assay reagent II (LARII) was dispensed into the required amount of clear 1.5 ml tubes. Medium was aspirated from transfected cells. The cells were washed twice in with 1 ml PBS and 100 µl 1X passive lysis buffer added. The plate was then placed on an orbital shaker, at 50 rpm for 15 minutes at room temperature. 20 µl of the cell lysate was then transferred into one of the 1.5 ml tubes containing the pre-dispensed LARII and mixed by pipetting. The tube was then placed into the Glomax luminometer (Turner BioSystems) and a 10 second measurement of the firefly luciferase activity taken. On prompting by the luminometer 100 µl of 1X Stop & Glo reagent was added and mixed, followed by a 10 second measurement of *renilla* luciferase activity. MMP1 promoter activity was measured as the ratio of firefly luciferase activity of MMP1 promoter construct versus *renilla* luciferase activity, divided by the ratio of firefly luciferase activity of pGL3-control plasmid versus *renilla* luciferase activity.

2.3.9 Statistical analysis of MMP1 promoter haplotype luciferase assays

Statistical analysis of MMP1 promoter haplotype luciferase assays was carried out by colleagues using a 2 tailed T-test to obtain p-values (193).

2.3.10 Statistical analysis of MMP8 promoter luciferase assays

MMP8 promoter SNP rs11225395 luciferase data p-values were obtained using a 2-tailed t-test.

2.3.11 Statistical analysis of VCAM-1 promoter luciferase assays

VCAM-1 promoter luciferase data pairwise p-values were obtained using a 2-tailed t-test. Analysis of Variance (ANOVA) was used to determine 2-tailed p values for any difference in treatments overall.

2.4 Biochemical investigations of the role of MMP8 in atherosclerosis

2.4.1 Angiotensin I cleavage by MMP8

To confirm the reported ability of MMP8 to cleave angiotensin I (Ang I) into angiotensin II (Ang II); Ang I cleavage by MMP8 was performed following the protocol of the reporting study (194). Ang I (Phoenix Pharmaceuticals) was incubated in TCNB (50 mmol/l Tris, 10mmol/l $CaCl_2$, 150 mmol/l NaCl, 0.05% Brij 35 (Sigma), pH 7.5) with human neutrophil purified MMP8 (Biomol International, SE-492) in the presence of 0.5mmol/l 4-aminophenylmercuric acetate (APMA) to activate proMMP8. The incubation was at 37°C for 20 hours at a substrate concentration of 25 µmol/l and an enzyme substrate ratio of 1:100; an uncleaved control was prepared by incubating angiotensin I in TCNB at 37°C for 20 hours without MMP8.

2.4.1.1 Analysis of cleavage products of Ang I by MMP8 via LC-MS/MS

Colleagues analysed the cleavage products and controls described above via liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS). The mass spectral data were processed into peak lists and searched against the Swiss Prot database. The relative intensity of the angiotensin peptides was calculated using the total ion count of each peptide.

2.4.2 Biochemical and animal investigations of the role of MMP8 in atherosclerosis

2.4.2.1 Animals

All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom). MMP8/ApoE double knockout mice (MMP8-/-ApoE-/-) and littermate controls (MMP8+/+ApoE-/-) were generated by crossing MMP8 knockout mice (122) with ApoE knockout mice (195, 196). Initially MMP8-/-apoE+/+ mice with a C57BL/6 background, (Jackson Laboratory, stock number 005514, strain name B6;129X1-Mmp8tm1Lotn/J) were crossed with MMP8+/+apoE-/- mice also of a C57BL/6 background (Jackson Laboratory, stock number 002052, strain name B6.129P2-Apoetm1Unc/J) to generate MMP8+/-/apoE+/- double heterozygous mice. The double heterozygous mice were bred to produce MMP8-/-/apoE-/- double knockout mice and MMP8+/+/apoE-/- littermate controls; the breeding was performed by the Jackson Laboratory (Bar Harbor, USA). From the age of 6 weeks male mice from the double knockout group and the MMP8 wild type littermate controls were fed a high fat, Western diet (Research Diet, D12108, containing 21% fat, 1.25% cholesterol and 0% cholate) for a total of 12 weeks.

2.4.2.2 Quantification of plasma angiotensin I with a competitive enzyme immunoassay kit (Phoenix Pharmaceuticals, EK-002-01)

Kit Components

1. 20x assay buffer concentrate (50ml).

2. 96 well immunoplate (1 plate).

3. Acetate plate sealer (APS), (3 pieces).

4. Primary antiserum (rabbit anti-peptide IgG) (1 vial).

5. Peptide standard (Ang I) (1 vial).

6. Biotinylated peptide (1 vial).

7. Streptavidin-horseradish peroxidase (SA-HRP) (30µl).

8. Positive control (2 vials).

9. Substrate solution (TMB) (12ml).

10. 2N HCl (15ml).

All kit components were allowed to come to room temperature for 30 minutes before starting. The 20x assay buffer was diluted in 950 ml of deionised H_2O to make 1x assay buffer solution. The standard peptide (1 mg Ang I) was diluted with 1 ml of 1x buffer solution and left for 10 minutes at room temperature to make a 1000 ng/ml stock standard peptide solution. Standards were made by serially diluting this solution as follows (table 2.6).

Table 2.6 Ang I standard dilution series.

Standard No.	Std. volume	1x Assay buffer	Concentration (ng/ml)
Stock	1000 µl	----	1000 ng/ml
#1	25 µl	975 µl	25 ng/ml
#2	200 µl of #1	800 µl	5 ng/ml
#3	200 µl of #2	800 µl	1 ng/ml
#4	200 µl of #3	800 µl	0.2 ng/ml
#5	200 µl of #4	800 µl	0.04 ng/ml

Both the primary antibody and the biotinylated peptide were each separately dissolved in 5 ml of 1x assay buffer and incubated at room temperature for 5 minutes. The positive control was rehydrated with 200 µl of 1x assay buffer and incubated at room temperature for 5 minutes. 2 wells of the immuno-plate were left empty as blank, 50 µl 1x assay buffer was added of a further two wells to provide a total binding reference. 50 µl of all standards, positive controls and prepared samples (at desired dilution) were added to the appropriate wells in duplicate. 25 µl of primary antibody was added to all

wells except the blank wells. 25 µl of biotinylated peptide was added to all wells except the blank wells, the plate sealed with the provided APS and incubated at room temperature for 2 hours at 400 rpm on an orbital shaker. 12 µl of SA-HRP was added to 12 ml of 1x assay buffer to make the SA-HRP solution. Each well was washed 4 times with 350 µl 1x assay buffer. 100 µl of SA-HRP solution was added to each well, the plate resealed and incubated at room temperature for 1 hour at 400 rpm on an orbital shaker. After incubation the plate was washed a further 4 times as above and 100 µl of TMB substrate solution added to each well and the plate sealed. Incubation with the TMB solution was 1 hour at room temperature in the dark. After 1 hour 100 µl of 2N HCl was added to each well and the absorbance read at 450 nm within 20 minutes. Values were calculated by plotting a standard curve on semi-log graph paper. Known concentrations of the standard were plotted on the log scale (X axis) versus O.D. reading on the linear scale (Y axis). Sample concentration was determined via interpolation of its O.D. As the assay is a competitive immunoassay relying on the displacement of biotinylated peptide by angiotensin I, the standard curve is of reverse sigmoidal shape, the lower angiotensin I concentration giving a higher intensity absorbance and the higher angiotensin I concentration giving low absorbance values.

2.4.2.3 Quantification of plasma angiotensin II with a competitive enzyme immunoassay kit (Phoenix Pharmaceuticals, EK-002-012)

The quantification of angiotensin II was identical in protocol to that of angiotensin I with the relevant components being substituted, such as the peptide standard (1 mg Ang II), the biotinylated peptide, the positive control and the primary antibody.

2.4.2.4 Analysis of other angiotensin forming enzymes

Tissue samples were prepared using a method adapted from a previous study (197). Aortas from MMP8+/+ApoE-/- or MMP8-/-ApoE-/- mice were cut into small pieces and immediately placed into ice cold 20 mM Na-phosphate buffer pH 7.4 in Precellys CK14 bead tubes, and lysed at 4°C at 2X 6500 rpm for 15 seconds in a Precellys24 homogeniser. The lysate from each aorta was transferred into a microfuge tube and centrifuged at 25,000g for 30 minutes at 4°C. The pellet was resuspended in 10 mM Na-phosphate buffer pH 7.4, containing 2 M KCl and 0.01% Nonidet P-40. The suspension was divided into 2 microfuge tubes and centrifuged at 25000g for 30 minutes at 4°C. The pellet from one tube was resuspended in 150 µl 10 mM Na-phosphate buffer pH 7.4, containing 2 M KCl and 0.01% Nonidet P-40. After storage at 4°C for 18 hours this suspension was subjected to analyses of chymase and cathepsin activity. The pellet from the other tube was resuspended in 150 µl 10 mM Na-phosphate buffer pH 7.4, containing 2 M KCl and 0.6% Nonidet P-40, and after storage at 4°C for 18 hours centrifuged again as above; the resulting supernatant was used in the ACE activity assay. The protein content of both the ACE sample preparation and the chymase/cathepsin sample preparation was ascertained via the Bradford method (198).

2.4.2.5 Quantification of protein in chymase/cathepsin sample preparations via the Bradford method

Reagents and materials

Dye Reagent Concentrate (Biorad, 500-006)

Optical microplate (96 well)

Quantification of protein in chymase/cathepsin sample preparations was via the Bradford method (198). Working dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts deionised water. This solution was then filtered to remove particulates. A set of bovine serum albumin (BSA; Sigma) standards (0.2 – 1.0 mg/ml) was prepared using 10 mM Na-phosphate buffer pH 7.4, containing 2 M KCl and 0.01% Nonidet P-40. 10 µl of each standard and sample solution were pipetted into separate microtiter plate wells, in duplicate. 200 µl of diluted dye reagent was added into each well; the samples were then mixed thoroughly using an orbital shaker. After 5 minutes incubation at room temperature absorbance values were read at 595 nm on a spectrophotometer (Dynex MRX Revelation). Sample protein concentrations were calculated from the standard curve in Microsoft Excel:

2.4.2.6 Quantification of protein in ACE sample preparations via the Bradford method

This was as that of the chymase/cathepsin protein quantification with some changes. BSA standards were made up in 10 mM Na-phosphate buffer pH 7.4, containing 2 M KCl and 0.1% Nonidet P-40. Prior to analysis ACE sample preparations were diluted 1 in 6 in 10 mM Na-phosphate buffer pH 7.4, containing 2 M KCl; this dilution was to prevent the relatively high Nonidet P-40 concentration of the above samples (0.6%) from interfering with the assay. Post analysis the protein values obtained were multiplied by 6 to account for the dilution factor.

2.4.2.7 Determination of ACE activity

ACE activity was determined with the use of fluorogenic peptide substrate V (Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH; R&D Systems). Using a final sample volume of 100 µl to measure ACE activity, 10 µl of Mus aorta extract sample was added to assay buffer that contained 50 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 6.5; Sigma) and chymostatin (Chymase Assay Kit #CS1140, Sigma) with or without the ACE inhibitor captopril (100 µM); chymostatin was used to exclude chymase and/or cathepsin activity from the assay. The reaction was started by the addition of 2.5 µl of the fluorogenic substrate (10 µM, final concentration). The reaction mixture was incubated on a Wallac Victor II spectrofluorometer at 37°C for 90. Fluorescence was measured at an excitation wavelength of 340 nm and emission wavelength of 400 nm. The assays for each sample were performed in duplicate. For each sample, ACE activity was calculated by subtracting the fluorescence reading in the assay with captopril from the fluorescence reading in the assay without captopril, and standardised against the protein concentration in the sample.

2.4.2.8 Determination of chymase/cathepsin activity

Chymase/cathepsin activity was determined with the use of the colorimetric substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide, Chymase Assay Kit, Sigma). Using a final sample volume of 100 µl to measure chymase/cathepsin activity, 10 µl of Mus aorta extract sample was added to assay buffer (A9606, Sigma) and captopril (100 µM) with or without the chymase/cathepsin inhibitor chymostatin (Chymase Assay Kit #CS1140, Sigma); captopril was used to exclude ACE activity from the assay. The reaction mixture was incubated at 37°C for 30min, and absorbance at 405nm was measured

using a spectrophotometer (Dynex MRX Revelation). The assays for each sample were carried out in duplicate. For each sample, enzymatic activity was calculated by subtracting the absorbance reading in the assay with chymostatin from the absorbance reading in the assay without chymostatin, and standardised against the protein concentration in sample

2.4.2.9 Quantification of Substance P with a competitive enzyme immunoassay kit (Substance P Correlate™ EIA Kit, 900-018)

Kit components:

1. 96 well goat anti-rabbit IgG microtiter plate (1 Plate)
2. Substance P EIA alkaline phosphatase conjugate (6 ml; blue)
3. Substance P EIA antibody (6 ml; yellow)
4. Assay Buffer (30 ml)
5. Wash buffer concentrate (30 ml)
6. Substance P standard (100000 pg/ml, 0.5 ml)
7. p-nitrophenyl phosphate (p-Npp), substrate (20 ml)
8. Stop solution, (5 ml)
9. Substance P assay layout sheet (1)
10. Acetate plate seal (1)

All kit components were allowed to come to room temperature for 30 minutes before starting. Standards (viable for 1 hour after preparation) were made up in the following manner: The 100,000 pg/mL substance P standard solution (room temperature) was diluted in 6 individual 1.5 ml microtubes. Volumes are detailed in table X; tubes were capped and vortexed thoroughly in between dilutions.

Table 2.7 Substance P standard dilution series

Standard No.	Std. volume	1x Assay buffer	Concentration (ng/ml)
Standard solution			100000 pg/ml
#1	100 µl of standard	900 µl	10000 pg/ml
#2	250 µl of #1	750 µl	2500 pg/ml
#3	250 µl of #2	750 µl	625 pg/ml
#4	250 µl of #3	750 µl	156.25 pg/ml
#5	250 µl of #4	750 µl	39.06 pg/ml
#6	250 µl of #5	750 µl	9.76 pg/ml

Wash buffer was prepared by adding 5 ml of wash buffer concentrate to 95 ml of deionised water. Assays were carried out as per manufacturer's instructions.

All wells were in duplicate. 50 µl of assay buffer was pipetted into the NSB (non-specific binding) and the B_0 (0 pg/ml standard) wells. 50 µl of the diluted standards (#1 - #6) were pipetted into the appropriate wells. 50 µl of prepared samples were added to the appropriate wells. A further 50 µl of assay buffer was added to the NSB wells. 50 µl of blue conjugate was added to all wells except total activity (TA) and blank wells (blank and TA wells had not had anything added at this point). 50 µl of yellow antibody was added to all wells, except the blank, TA and NSB wells; the plate sealed with the provided APS and incubated at room temperature for 2 hours at 400 rpm on an orbital shaker. Each well was washed 3 times with 400 µl of wash buffer; after washing each well was aspirated and the plate tapped firmly upside-down to remove residual wash buffer. 5 µl of the blue conjugate was added to the TA wells. 200 µl of the p-Npp substrate solution was added to every well and the plate incubated at room temperature for 1 hour without shaking. After 1 hour 50 µl of stop solution was added to all wells. The optical density (OD) was then read on a spectrophotometer (Dynex MRX Revelation) at 405nm, correcting at 570nm, also subtracting the blank wells.

The average net OD for each sample and standard was calculated by subtracting the average NSB OD from each. The binding for each of the standards (as a percentage) was calculated using the following formula:

$$\text{Percentage binding} = \left(\frac{Net\ (standard)OD}{Net\ B0\ OD}\right) 100$$

A standard curve (as an approximate straight line between points) was then drawn on Logit-Log paper plotting percentage bound against the known concentration of the standards. Sample values were determined by interpolation.

2.4.2.9.1 Extraction of substance P from Mus mesentery

Required items:

CK14 bead tubes (Precellys)

C18 SEP-COLUMN (Phoenix Pharmaceuticals)

Precellys24 homogeniser (Precellys)

Savant SpeedVac system (Thermo Scientific)

Elution Solvents:

- Buffer A: 1% trifluoroacetic acid (TFA) (HPLC grade, Sigma) in H_2O.
- Buffer B: 60% acetonitrile (HPLC grade; Sigma) in 1% TFA.

Buffers:

- 20 mM Na-phosphate buffer pH 7.4
- 1X assay buffer from (Substance P Correlate™ EIA Kit, 900-018)

Samples of Mus mesentery were cut into small pieces and immediately placed into ice cold 20 mM Na-phosphate buffer pH 7.4 (10 volumes w/v, at least 300 µl in CK14 bead tubes; Precellys). The samples were lysed at 4°C in a Precellys24 homogeniser (2x 6500

rpm for 15 seconds). The CK14 tube contents (minus the ceramic beads) were transferred to 1.5 ml microfuge tubes and centrifuged at 16400 rpm (25000g) for 30 minutes at 4°C. For each sample a SEP-COLUMN was equilibrated by washing with buffer B (1ml, once) followed by buffer A (3 ml, 3 times). The sample supernatant was acidified by mixing 1:1 with 1% trifluoroacetic acid in H_2O and loaded onto the pre-treated column. Once the sample was bound to the column matrix, the column was washed slowly with buffer A (3 ml, twice) and the wash discarded. The peptide was eluted slowly with buffer B (3 ml, once) into a polypropylene tube and then divided into 4x 1.5 ml microtubes. The eluant was evaporated to dryness by SpeedVac.

Samples were reconstituted with 1x assay buffer (from Correlate™ EIA Kit) before quantification. Protein concentration for sample normalisation was performed using the Bradford method (198)

2.4.2.10 Quantification of protein substance P mesentery sample preparations via the Bradford method

This was the same as section 2.4.2.5 with the substitution of 1x assay buffer for the 10 mM Na-phosphate buffer pH 7.4, containing 2 M KCl and 0.01% Nonidet P-40.

2.4.2.9 Measurement of the extent of atherosclerosis

The characterisation of atherosclerotic lesions was performed by colleagues. The extent of atherosclerotic lesions was analysed in both the double knockout (n = 10) group and the controls (n = 10) by en face staining with oil red of aortas (199).

2.4.2.10 Measurement of plasma cholesterol

The plasma cholesterol concentrations of the double knockout group and the controls were compared using a Cholesterol/Cholesteryl Ester Quantitation Kit (Calbiochem, 428901). The principal of this assay is as follows; enzymes provided in the kit, cholesterol esterase and cholesterol oxidase, react with the different forms of cholesterol in the sample forming hydrogen peroxide (H_2O_2) as a by-product. Hydrogen peroxide then reacts with the cholesterol probe to form a substance that can be detected at 570 nm.

Kit components

- Cholesterol Reaction Buffer (1 bottle, 25 ml).
- Cholesterol Probe (1 vial, lyophilised).
- Dimethylsulfoxide (400 µl DMSO).
- Enzyme Mix (1 vial, lyophilised).
- Cholesterol Esterase (1 vial, lyophilised).
- Cholesterol Standard (1 vial, 100 µl, supplied at 5 µg/µl).

All kit components were allowed to come to room temperature for 20 minutes before starting. The cholesterol probe was prepared by dissolving it in 220 µl DMSO. The cholesterol esterase was reconstituted by the addition of 220 µl cholesterol reaction buffer. The enzyme mix was reconstituted by the addition of 220 µl cholesterol reaction buffer. The cholesterol standard was diluted with 180 µl cholesterol reaction buffer to produce a stock solution of 0.5 µg/µl and allowed to sit at room temperature for 5 minutes. A set of standard duplicates were made up in wells (table 2.7).

Table 2.8. Cholesterol standard dilution series

Volume of 0.5 µg/µl diluted cholesterol standard	Cholesterol reaction buffer	Final amount of cholesterol per well
0 µl	50 µl	0 µg
4 µl	46 µl	2 µg
8 µl	42 µl	4 µg
12 µl	38 µl	6 µg
16 µl	34 µl	8 µg
20 µl	30 µl	10 µg

Enough reaction mix was prepared for the required amount of wells; in a single well the constituents of the added reaction mix were:

44 µl Cholesterol Reaction Buffer

2 µl Cholesterol Probe

2 µl Enzyme Mix

2 µl Cholesterol Esterase

2 µl of each sample were made up to 50 µl addition of 48 µl cholesterol reaction buffer. 50 µl of the reaction mix was added to each well followed by incubation for 1 hour at 37°C away from the light. Absorbances were read on a microplate reader at 570 nm. The no cholesterol control value was subtracted from all samples. Samples were calculated from the standard curve.

2.4.2.11 Measurement of plasma triglyceride

The plasma triglyceride concentrations of the double knockout group and the controls were compared using a Serum Triglyceride Determination Kit (Sigma, TR0100). Triglycerides within the sample are hydrolysed to glycerol. Glycerol is then

phosphorylated by glycerol kinase to form glycerol-1-phosphate. Glycerol-1-phosphate is then oxidised by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. Hydrogen peroxide is then coupled with 4-aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine by peroxidase to produce a quinone-imine dye. The amount quinone-imine dye, detectable at 540 nm, is directly proportional to the triglyceride concentration in the sample.

Kit Components

- Triglyceride Reagent (10 ml - T2449)
- 250,000 units/l Lipase (microbial), 0.05% Sodium azide.
- Free Glycerol Reagent (40 ml - F6428)
- 0.75 mM ATP, 3.75 mM Magnesium salt, 0.188 mM 4-Aminoantipyrine, 2.11 mM N-Ethyl-N-(3-sulfopropyl) m-anisidine, sodium salt, 1,250 units/l Glycerol Kinase (microbial), 2,500 units/l Glycerol Phosphate Oxidase (microbial), 2,500 units/l Peroxidase (horseradish) Buffer, pH 7.0 ± 0.1, 0.05% Sodium azide.
- Glycerol Standard Solution, 2.5 mg/ml equivalent triolein concentration (G7793)

Note: All H_2O 18Ω

All reagents were allowed to attain room temperature before reconstitution. The free glycerol reagent was reconstituted by adding 40 ml of H2O, the triglyceride reagent by adding 10 ml. both reagents were mix by inversion in stoppered vials. The triglyceride working reagent was prepared by mixing reconstituted free glycerol reagent with

reconstituted triglyceride reagent in a 4:1 ratio and storing in an amber bottle to protect it from the light. The triglyceride reagent blank was prepared by mixing the reconstituted free glycerol reagent with H2O in a 4:1 ratio. 198 µl of triglyceride working reagent was added to each well. 2 µl of water or glycerol standard or sample was added to each well (all blanks, standards, samples in duplicate). True blanks, their absorbance to be subtracted from all other wells were made with 200 µl of H2O. The contents of the microplate were then mixed and incubated at 37°C for 5 minutes; absorbance was read at 540 nm. Total triglyceride was calculated using the following calculation:

$$\frac{(A_{sample} - A_{Blank}*)}{(A_{Standard} - A_{Blank}*)} \text{ x Concentration of Standard}$$

* Blank in the calculation refers to the one containing triglyceride working reagent and water.

2.4.2.12 Quantification of mouse plasma soluble VCAM-1 with Quantikine Mouse sVCAM-1 Immunoassay (R&D Systems, MVC00)

This assay employs the quantitative sandwich enzyme immunoassay technique. A specific monoclonal antibody is bonded onto the microplate solid surface. Samples are added and any mouse sVCAM-1 present is bound by the immobilized antibody. A secondary specific enzyme linked antibody is then added. Following the addition of substrate a colour change occurs, the intensity in proportion to the amount of sample sandwiched between the two antibodies.

Kit Components

- Mouse sVCAM-1 Microplate ((12 strips of 8 wells) Coated with a monoclonal antibody specific for mouse sVCAM-1)
- Mouse sVCAM-1 Conjugate Concentrate (650 µl of concentrated solution containing monoclonal antibody against mouse sVCAM-1 conjugated to horseradish peroxidise)
- Type 2 Conjugate (12.5 ml of diluent for diluting the conjugate concentrate.
- Mouse sVCAM-1 Standard (3 vials of 40 ng lyophilized))
- Mouse sVCAM-1 Control (3 vials, lyophilized)
- Assay Diluent RD1-47 (12.5 ml of a buffered protein solution)
- Calibrator Diluent RD5-26 Concentrate (21 ml of a concentrated buffered protein solution)
- Wash Buffer Concentrate (50 ml of a concentrated solution of a buffered surfactant)
- Colour Reagent A (12.5 ml of stabilised hydrogen peroxide)
- Colour Reagent B (12.5 ml of stabilised chromogen (tetramethylbenzidine))
- Stop Solution (23 ml of a diluted hydrochloric acid solution)
- Plate Covers (4 adhesive plate seals)

All kit components were allowed to come to room temperature for 30 minutes before starting. The mouse sVCAM-1 control was reconstituted in 1 ml of deionised H_2O. 0.5 ml of conjugate concentrate was added to 11 ml of conjugate diluent to make 1x working conjugate. Wash buffer was prepared by adding 25 ml of wash buffer

concentrate to 600 ml of deionised H_2O. 20 ml of calibrator diluent RD5-26 Concentrate was added 60 ml of deionised H_2O to prepare 80 ml of 1x calibrator diluent RD5-26. Mouse sVCAM-1 standard was reconstituted with 2.0 ml of 1x calibrator diluent RD5-26 to produce a stock solution of 20 ng/ml and allowed to sit at room temperature for 5 minutes. A set of standards were made by serially diluting mouse sVCAM-1 standard in 1x calibrator diluent RD5-26 as follows (table 2.8).

Table 2.8. Mouse sVCAM-1 standard dilution series

Standard No.	Std. volume	1x Diluent RD5-26	[ng/ml]
Stock	200 µl	----	20 ng/ml
#1	200 µl	200 µl	10 ng/ml
#2	200 µl of #1	200 µl	5 ng/ml
#3	200 µl of #2	200 µl	1.25 ng/ml
#4	200 µl of #3	200 µl	0.62 ng/ml
#5	200 µl of #4	200 µl	0.31 ng/ml
#6	----	200 µl	0 ng/ml

Mouse plasma samples were diluted 50 fold in RD5-26. 50 µl of assay diluent RD1-47 was added to each well. 50 µl of all standards, controls and prepared samples were added to the appropriate wells in duplicate, sealed and incubated at room temperature for 3 hours. Each well was washed 5 times with 400 µl wash buffer and the excess liquid blotted away on clean paper towels. 100 µl of mouse sVCAM-1 conjugate was added to each well, the plate sealed and incubated for 1 hour at room temperature. The plate was washed 5 times as above. The substrate solution was made up by combining colour reagents A and B in equal volumes 15 minutes prior to use. 100 µl of substrate solution was added to each well and the plate incubated at room temperature for 30 minutes away from the light. The reaction was halted with the addition of 100 µl of stop solution and the absorbance read on a microplate reader at 450 nm, with a correction wavelength of 540 nm, within 30 minutes. Values were calculated by plotting a standard

curve; known concentrations of the standard were plotted on the log scale (X axis) versus O.D. reading on the log scale (Y axis). Sample concentration was determined via its O.D. and its intersection with the curve and the Y axis.

2.4.3 Statistical analysis of investigations into the role of MMP8 in atherosclerosis

The differences between MMP8-/-ApoE-/- double knockout mice and MMP8+/+ApoE-/- littermate control mice in terms of plasma levels of Ang I, Ang II, soluble VCAM-1, substance P, cholesterol and triglycerides were assessed by 2-tailed t-Test. Colleagues also used the t-Test to ascertain differences between the 2 groups in the extent and content of atherosclerotic lesions, including macrophage, smooth muscle cell, collagen, Ang II and VCAM-1, as well as blood pressure, leukocyte rolling and adhesion, and mean fluorescence intensity in flow cytometric analysis.

3. Results

3.1 Investigation into the effects of MMP1 promoter haplotypes on gene expression in cancer cells

3.1.1 Linkage disequilibrium between *MMP1* promoter polymorphisms

A group of 276 unrelated individuals of British Caucasian phenotype were genotyped for seven *MMP1* promoter polymorphisms, to measure LD and identify common haplotypes. The promoter polymorphisms had previously been identified by Pearce *et al* in 2005 (24) by the sequencing of the 1.9 kb *MMP1* promoter in 30 unrelated British Caucasians (from the same cohort as above). The reference SNP ID, the position relative to the start of transcription and the substitution for each SNP is shown in table 3.1.

Table 3.1.

rs number	Position relative to the start of transcription (bp)	Substitution/deletion
rs494379	-320	C>T
rs514921	-340	C>T
rs475007	-422	T>A
rs1144393	-519	A>G
rs498186	-755	G>T
rs473509	-839	G>A
rs1799750	-1607	GG>G

Analysis of the genotyping data with Haploview showed strong but incomplete LD between the seven SNPs (Figure 3.1).

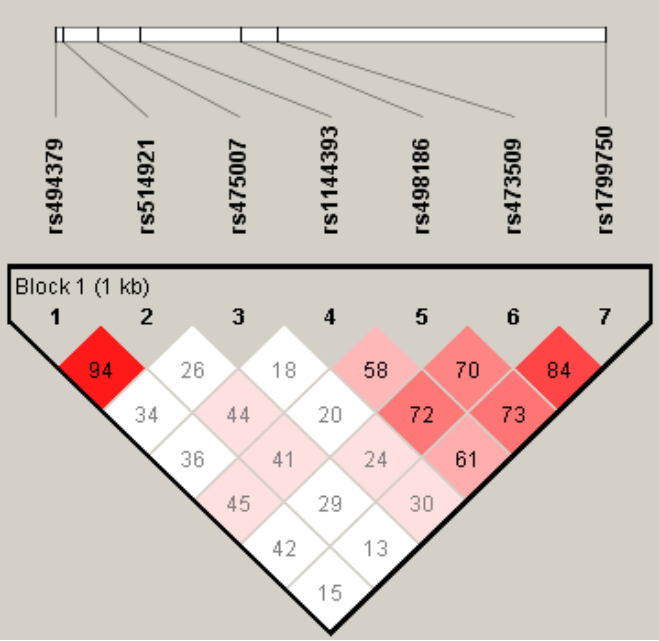

FIGURE 3.1. Linkage disequilibrium plot. Coefficients (D') of pairwise linkage disequilibrium coefficients between the seven polymorphisms in the MMP1 gene promoter.

At the time of analysis genotype data for only three of these SNPs was available on the HapMap database rs498186, rs475007, and rs514921 and are shown in figure 3.2 to be located in a recombination hotspot. This remains the case with the latest available data, submitted June 2008.

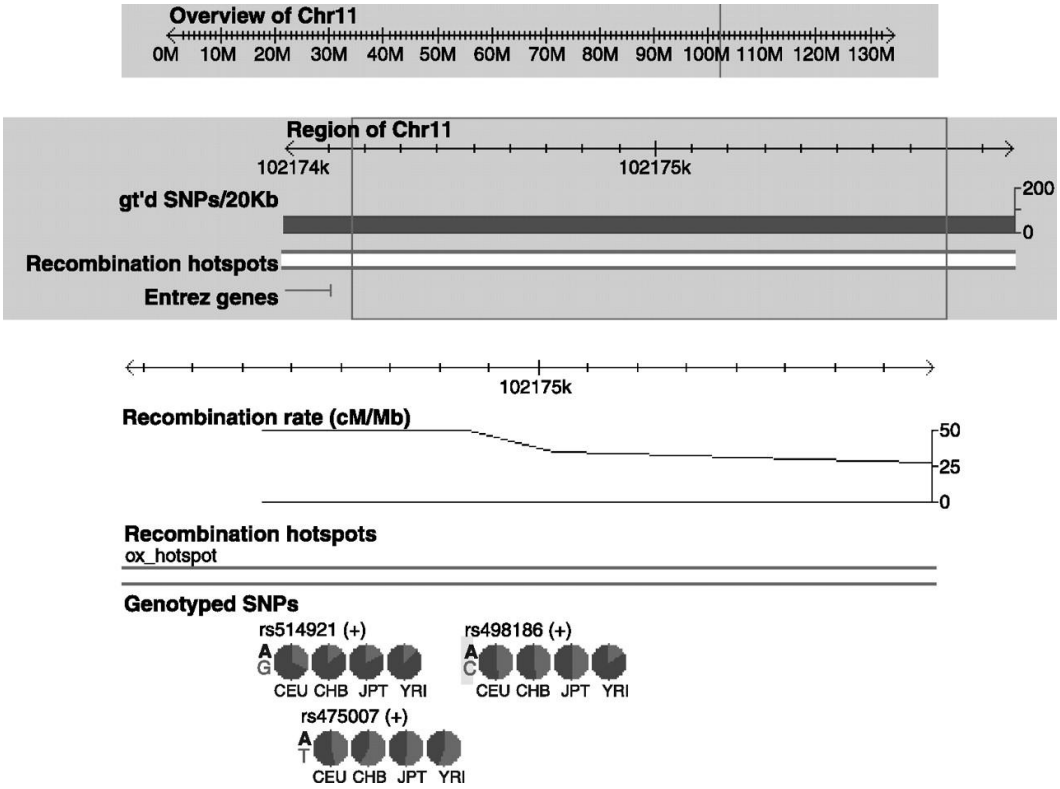

FIGURE 3.2. Recombination rates. Information obtained from the HapMap database showing a recombination hotspot around the MMP1 gene rs498186 (-755G>T) polymorphism (shaded).

3.1.2 The 10 most frequent haplotypes of the MMP1 promoter polymorphisms

Haplotype analyses using Haploview, THESIAS, and PHASE programs showed 10 common haplotypes (freq of > 2.5%; table 3.2)

Table 3.2.

Haplotype	THESIAS	Haploview	PHASE
G-A-T-G-A-T-T	0.141	0.144	0.170
GG-G-G-A-T-T-T	0.112	0.116	0.129
G-A-T-G-T-T-T	0.105	0.103	0.092
GG-G-G-A-T-C-T	0.100	0.091	0.100
GG-G-T-A-T-T-C	0.060	0.059	0.065
GG-G-G-A-A-C-T	0.045	0.046	0.053
GG-G-G-A-A-T-T	0.039	0.036	0.042
G-G-T-A-A-C-T	0.032	0.032	0.031
GG-G-G-A-T-T-C	0.029	0.027	0.031
G-A-T-G-A-C-T	0.028	0.026	0.025

NOTE: The table shows the 10 most common haplotypes and their frequencies estimated using the THESIAS, Haploview, and PHASE programs. The haplotypes were derived from the rs1799750, rs473509, rs498186, rs1144393, rs475007, rs514921, and rs494379 SNPs, with G-A-T-G-A-T-T denoting a haplotype encompassing rs1799750-G, rs473509-A, rs498186-T, rs1144393-G, rs475007-A, rs514921-T, and rs494379-T, and so on. All haplotypes retain the same SNP order.

3.1.3 MMP1 promoter haplotype luciferase activity

As variation in the MMP1 gene has reportedly been associated with several types of cancer (25-29); the potential effects of the 10 MMP1 promoter haplotypes on expression in cancer cell lines was tested by luciferase reporter assay. In all eight cancer cell lines were tested with MMP1 promoter haplotypes, two melanoma cell lines (A2058, and A375), two breast cancer cell lines (MDA-MB-231 and MCF7), two lung cancer cell lines (A549 and H69), and two colorectal cancer cell lines (HT-29 and SW620). They were transiently transfected with pGL3-basic vectors into which MMP1

promoters of the corresponding haplotype had been cloned, and subjected to luciferase assay. The data here represents 3 independent experiments; furthermore in each experiment samples and controls were in triplicate.

Figure 3.3.

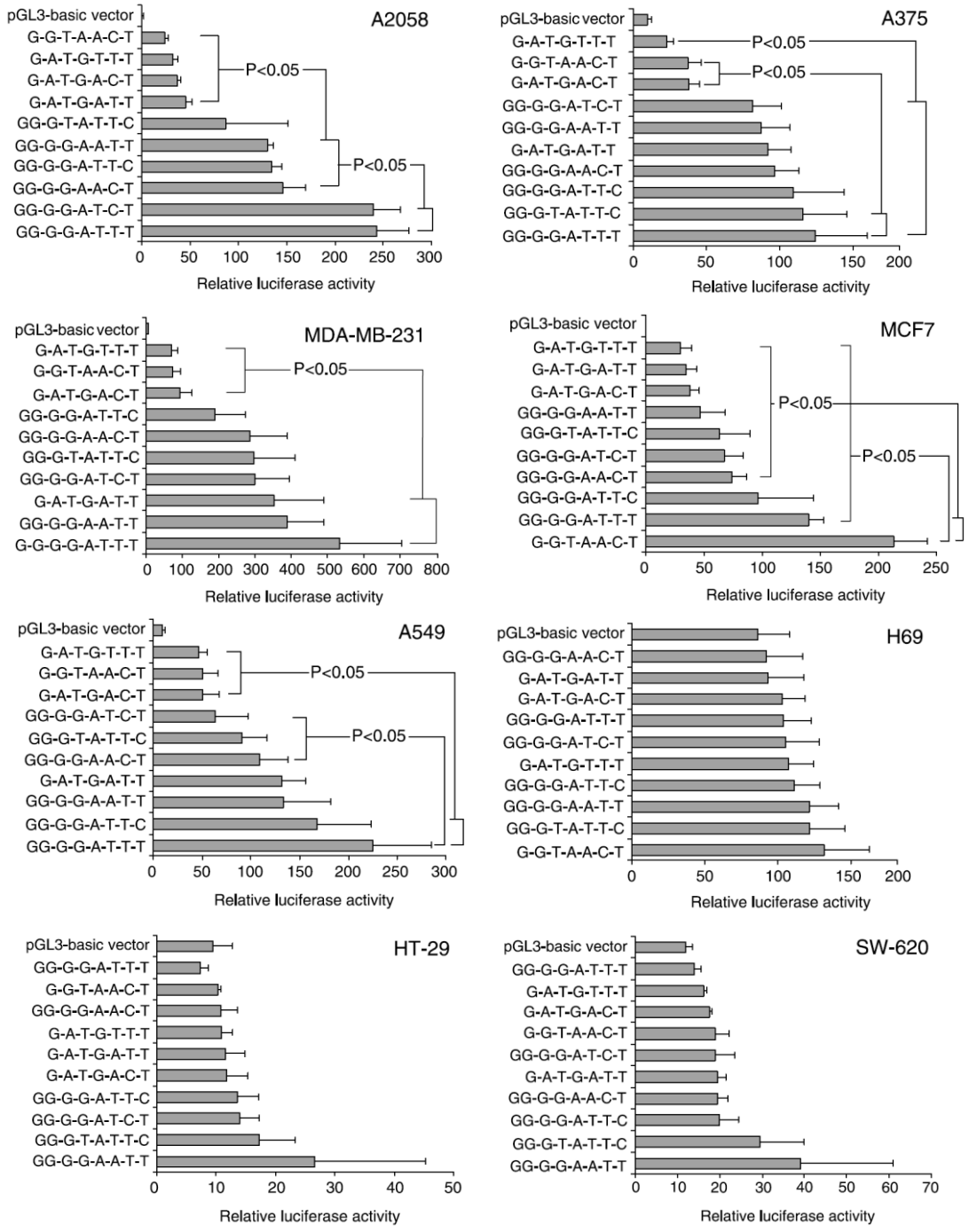

FIGURE 3.3. MMP1 gene promoter activity in cancer cells. Columns, relative transcriptional activity of the 10 differentMMP1 promoter haplotypes in A2058 (melanoma), A375 (melanoma), MDA-MB-231(breast cancer), MCF7 (breast cancer), A549 (lung cancer), H69 (lung cancer), HT-29 (colorectal cancer), and SW-620 (colorectal cancer) cells. Promoter activities were measured by luciferase assays as described in Materials and Methods. pGL3-basic vector columns, mean background luciferase expression levels from three independent experiments; bars, SD.

In the melanoma cell line A2058 two of the haplotypes containing the 2G allele of the rs1799750 polymorphism (GG-G-G-A-T-T-T and GG-G-G-A-T-C-T) had at least four-fold higher activity than haplotypes with the 1G allele of rs1799750. The other 2G containing haplotypes (GG-G-G-A-A-C-T, GG-G-T-A-T-T-C, GG-G-G-A-A-T-T, and GG-G-G-AT- T-C) had two to three fold higher promoter activity than the 1G containing haplotypes. Significant differences between haplotypes with the rs1799750 2G allele were that haplotypes GG-G-G-A-T-T-T and GG-G-G-A-T-C-T had approximately two fold higher activities than the others (Fig. 3.3).

In A375, the second melanoma cell line tested, there was less than two fold higher activity in haplotypes containing the 2G allele of rs1799750 than those containing the 1G allele with the exception of the G-A-T-G-A-T-T haplotype which had a had a similar activity to the 2G containing haplotypes (Fig. 3.3)

The breast cancer cell line MDA-MB-231 showed similar results to A375 melanoma cells in that there was less than two fold higher activity in haplotypes containing the 2G allele of rs1799750 than those containing the 1G allele with the exception of the G-A-T-G-A-T-T haplotype which had a had a similar activity to the 2G containing haplotypes (Fig. 3.3)

In MCF7, another breast cancer cell line, the highest promoter activity was in the rs1799750 1G allele containing haplotype G-G-T-A-A-C-T followed by the GG-G-G-A-T-T-T haplotype. The promoter strength of the remaining haplotypes was one and a half to four fold lower than the G-G-T-A-A-C-T and GG-G-G-A-T-T-T haplotypes (Fig. 3.3).

Once again in the A549 lung cancer cell line as in A2058, A375, and MDA-MB-231 cells the GG-G-G-A-T-T-T haplotype had the highest promoter activity. There was also a trend, though not significant of higher activity in rs1799750 2G containing haplotypes along with the G-A-T-G-A-T-T haplotype compared to other rs1799750 1G allele containing haplotype.

There were no significant differences between the haplotypes when tested in H69 lung cancer cells or the 2 colorectal cell lines (HT-29 and SW620), although contrasting to all the other cell lines (A2058, A375, MCF7, MDA-MB-231, and A549) the empty pGL3-basic vector had similar expression levels to pGL3 vectors containing the MMP1 promoter sequences.

The above data illustrate a potential for haplotypic effects on the MMP1 promoter to influence cancer through differential gene expression.

In the table below (Table 3.3) putative transcription factor binding sites are shown at each of the SNP loci. The in silico analysis shows the putative sites that might be gained or lost dependent on the allele present. Libraries of transcription factor binding sites and the algorithms used to predict them are improving all the time, but ultimately each site needs to be confirmed experimentally in the relevant cell or tissue types.

Table 3.3. Results of in silico search for putative transcription factor binding sites using the Genomatix SNPinspector program

Position	rsID	Allele	Transcription factor	Strand	Core similarity	Matrix similarity
-1607	rs1799750	GG	Ecotropic viral integration site 1 encoded factor, amino-terminal zinc finger domain	(-)	1	0.958
		G	GATA-binding factor 3	(-)	1	0.958
			Brn-2, POU-III protein class	(-)	0.933	0.886
-839	rs473509	G	PAX-5 B cell – specific activator protein	(-)	1	0.733
		A	PAX-1	(+)	0.75	0.626
			Thyrotrophic embryonic factor/hepatic leukaemia factor	(+)	0.784	0.802
-755	rs498186	G	Neuron-specific olfactory factor	(+)	1	0.9
		T	—			
-519	rs1144393	A	Pancreas transcription factor 1, heterotrimeric transcription factor	(+)	0.857	0.791
		G	Nuclear factor 1	(-)	1	0.987
-422	rs475007	T	Myoblast determining factor	(-)	1	0.983
			PAX-3 binding site	(+)	0.78	0.779
			Atp1a1 regulatory element binding factor 6	(+)	1	0.985
		A	—			
-340	rs514921	C	Hepatic nuclear factor 4	(+)	0.75	0.762
		T	Abd-B – like homeodomain protein Hoxb-9	(+)	1	0.94
-320	rs494379	C	—			
		T	Grainyhead-like 3	(+)	1	0.879

3.2 Investigation of MMP8 in relation to breast cancer

3.2.1 Characteristics of Leuven breast cancer study patients

The characteristics of patients of the Leuven breast cancer study, as characterised by colleagues in Leuven are as follows. The median age of patients at diagnosis was 56, ranging from 33-86. Of a total of 140, there were 53 premenopausal and 87 postmenopausal patients. In 68 patients the tumour size was defined as small (≤2 cm) and large (>2 cm) in 72 patients. 14 patients had well differentiated tumours, 52 patients had moderately differentiated tumours and 74 had poorly differentiated tumours. Lymph node involvement was found in 79 patients, and 61 patients were negative for lymph node involvement. Under the tumour node metastasis (TNM) staging system (American Joint Committee on Cancer) there were 39 patients classified with stage I, 74 were classified with stage II, and 27 with stage III.

3.2.2 Genotyping of MMP8 in relation to breast cancer in the Leuven cohort

Leuven breast cancer study patients were genotyped for nine tagging SNPs in and around the *MMP8* gene. SNP selection using HapMap data from January 2006 and the Tagger algorithm (168) is described in the methods section. There was no association found between any of the SNPs and tumour size, tumour grade, or the subtypes of luminal A, luminal B, basal-like, Her2+/ER-; Table 3.4. There was association found between four SNPs and lymph node metastasis. The minor alleles of these SNPs were found to have lower frequencies in patients with lymph node metastasis; the T allele of the promoter SNP rs11225395, the G allele of the non-synonymous SNP, in exon 2,

rs1940475, the A allele of the intron 4 SNP rs1892886, and the A allele of rs1276284, from the 3' downstream region of the *MMP8* gene (P = 0.02, P = 0.03, P = 0.03, and P = 0.03, respectively, Table 3.5).

Table 3.4. MMP8 genotypes and clinicopathological characteristics (other than lymph node status) of breast cancer patients

	all	rs10895353			p*	rs7943404			p*	rs11225395			p*	rs1320632		p*	rs1940475			p*
		AA	AG	GG		AA	AG	GG		CC	CT	TT		TT	CT		TT	CT	CC	
age at diagnosis, median	56	59	54	44	**0.13**	59	56	59	**0.56**	58	56	61	**0.15**	58	57	**0.89**	58	55	61	**0.12**
tumour size																				
<= 2 cm	68	50	16	0	**0.53**	29	26	13	**0.28**	25	26	17	**0.36**	58	10	**0.89**	22	25	21	**0.33**
> 2 cm	72	55	14	1		23	28	21		31	29	11		62	10		30	27	15	
grade																				
well/moderate	66	51	14	0	**0.62**	28	24	14	**0.45**	25	24	16	**0.47**	55	11	**0.45**	22	22	22	**0.15**
poor	74	54	16	1		24	30	20		31	31	12		65	9		30	30	14	
tumour subtype																				
luminal A	114	87	22	1	**0.54**	41	46	27	**0.81**	44	47	22	**0.29**	97	17	0.19	41	44	29	**0.48**
luminal B	11	7	4	0		3	4	4		6	4	1		11	0		6	4	1	
basal-like	10	6	4	0		5	3	2		4	4	2		9	1		4	3	3	
Her2+/ER-	5	5	0	0		3	1	1		2	0	3		3	2		1	1	3	

note: results tabulated as patients per genotype
* Pearson Chi square test

Table 3.4 continued. MMP8 genotypes and clinicopathological characteristics (other than lymph node status) of breast cancer patients

	all	rs1892886			p*	rs17099436		p*	rs2508383			p*	rs1276284			p*
		AA	AT	TT		AA	AT		GG	AG	AA		GG	AG	AA	
age at diagnosis, median	56	57	58	65	0.23	58	60	**0.46**	58	58	60	**0.9**	57	59	58	**0.66**
tumour size																
<= 2 cm	68	36	25	5	0.63	62	6	**0.92**	46	18	3	**0.86**	31	28	9	**0.55**
> 2 cm	72	43	25	3		66	6		50	18	2		38	23	10	
grade																
well/moderate	66	37	24	5	0.7	60	6	**0.84**	39	22	3	**0.09**	30	25	11	**0.52**
poor	74	42	26	3		68	6		57	14	2		39	26	8	
tumour subtype																
luminal A	114	66	40	6	0.75	104	10	**0.62**	75	32	4	**0.31**	56	42	15	**0.37**
luminal B	11	6	5	0		10	1		10	1	0		6	5	0	
basal-like	10	5	3	1		10	0		8	2	0		6	2	2	
Her2+/ER-	5	2	2	1		4	1		3	1	1		1	2	2	

note: results tabulated as patients per genotype
* Pearson Chi square test

Table 3.5. MMP8 SNPs in relation to lymph node metastasis in the Leuven Breast Cancer Study

Genotype	Number (%)		P*	P**	Allele	Number (frequency)		OR (95% CI)	P***
	LN+	LN-				LN+	LN-		
rs10895353									
AA	56 (73.7)	49 (81.7)	0.23	0.17	A	132 (0.87)	108 (0.90)	1.4 (0.6, 2.9)	0.42
AG	20 (26.3)	10 (16.7)			G	20 (0.13)	12 (0.10)		
GG	–	1 (1.7)							
rs7943404									
TT	26 (32.9)	26 (42.6)	0.27	0.12	T	82 (0.52)	76 (0.62)	1.5 (1.0, 2.5)	0.08
CT	30 (32.9)	24 (39.3)			C	76 (0.48)	46 (0.38)		
CC	23 (39.1)	11 (18.0)							
rs11225395									
CC	36 (46.2)	20 (32.8)	0.09	0.04	C	103 (0.66)	64 (0.52)	0.6 (0.4, 0.9)	**0.02**
CT	31 (39.7)	24 (39.3)			T	53 (0.34)	58 (0.48)		
TT	11 (14.1)	17 (27.9)							
rs1320632									
AA	69 (87.3)	51 (83.6)	0.53	0.53	A	148 (0.94)	112 (0.92)	0.8 (0.3, 1.9)	0.55
AG	10 (12.7)	10 (16.4)			G	10 (0.06)	10 (0.08)		
rs1940475									
AA	35 (44.3)	17 (27.9)	0.12	0.5	A	97 (0.61)	59 (0.48)	0.6 (0.4, 1.0)	**0.03**
AG	27 (34.2)	25 (41.0)			G	61 (0.39)	63 (0.52)		
GG	17 (21.5)	19 (31.1)							
rs1892886									
TT	51 (67.1)	28 (45.9)	0.04	0.03	T	123 (0.81)	85 (0.70)	0.5 (0.3, 0.9)	**0.03**
AT	21 (27.6)	29 (47.5)			A	29 (0.19)	37 (0.30)		
AA	4 (5.3)	4 (6.6)							
rs17099436									
TT	73 (92.4)	55 (90.2)	0.64	0.64	T	152 (0.96)	116 (0.95)	0.8 (0.2, 2.4)	0.65
AT	6 (7.6)	6 (9.8)			A	6 (0.04)	6 (0.05)		
rs2508383									
CC	55 (70.5)	41 (69.5)	0.73	0.9	C	131 (0.84)	97 (0.82)	0.9 (0.5, 1.7)	0.7
CT	21 (26.9)	15 (25.4)			T	25 (0.16)	21 (0.18)		
TT	2 (2.6)	3 (5.1)							
rs1276284									
GG	47 (59.6)	22 (36.7)	0.02	0.04	G	116 (0.73)	73 (0.61)	0.6 (0.3, 0.9)	**0.03**
AG	22 (27.8)	29 (48.3)			A	42 (0.27)	47 (0.39)		
AA	10 (12.7)	9 (15.0)							

Note: LN+, patients with lymph node metastasis; LN-, patients without lymph node metastasis
OR, odds ratio for minor allele
P*, p value for genotypic Pearson chi squared test
P**, p value for trend test
P***, p value for allelic chi squared test

3.2.3 MMP8 haplotypes in relation to lymph node metastasis in the Leuven Breast Cancer Study

There were four haplotypes present in patients of the Leuven Breast Cancer Study with a frequency > 5% derived from the from the nine *MMP8* SNPs. Using the most common haplotype A-C-C-A-A-T-T-C-G (Haplotype 1) as the reference, the second most common haplotype A-T-T-A-G-A-T-C-A (Haplotype 2) was associated with lower odds ratio [0.52 (95% CI 0.29 – 0.96)] for lymph node metastasis (p=0.037, Table 3.6). There was not a statistically significant association between the lymph node metastasis and the other two major haplotypes G-T-C-A-A-T-T-C-G (Haplotype 3) and A-T-T-G-G-T-T-T-A (Haplotype 4), Table 3.6.

Table 3.6. MMP8 haplotypes in relation to lymph node metastasis in the Leuven Breast Cancer Study

Haplotype No.	Haplotype composition*	Haplotype frequencies		Odds ratio (95% CI), p-value**
		LN+	LN-	
Haplotype 1	A-C-C-A-A-T-T-C-G	0.45	0.36	1.00 (reference)
Haplotype 2	A-T-T-A-G-A-T-C-A	0.18	0.30	0.52 (0.29 - 0.96), p=0.04
Haplotype 3	G-T-C-A-A-T-T-C-G	0.13	0.09	1.29 (0.52 – 3.22), p=0.58
Haplotype 4	A-T-T-G-G-T-T-T-A	0.06	0.08	0.75 (0.29 – 1.96), p=0.57

Note: LN+, patients with lymph node metastasis; LN-, patients without lymph node involvement.

*Haplotypes deriving from the following SNPs in order: rs10895353; rs7943404, rs11225395, rs1320632, rs1940475, rs1892886, rs17099436, rs2508383 and rs1276284.

**p-values were obtained using the stochastic EM algorithm

3.2.4 Genotyping of MMP8 in relation to the occurrence of breast cancer in the Strangeways study

Strangeways study patients and controls were genotyped for nine tagging SNPs in and around the MMP8 gene. SNP selection was as with the Leuven study. There was association found between 3 SNPs and breast cancer. The minor alleles of these SNPs were found to have higher frequencies in patients with breast cancer compared to controls; the T allele of the promoter SNP rs11225395, the A allele of the intron 4 SNP rs1892886, and the A allele of rs1276284, from the 3' downstream region of the MMP8 gene ($p = 0.04$, $p = 0.01$, and $p = 0.001$, respectively, Table 3.7).

Table 3.7. MMP8 SNPs in relation to breast cancer in the Strangeways Breast Cancer Case Control Study

Genotype	Number		p*	p**	Allele	Number (frequency)		OR (95% CI)	P***
	Case	Controls				Case	Controls		
rs10895353									
AA	1864 (85.1)	1894 (83.4)	0.25	0.18	A	4031 (0.92)	4142 (0.91)	0.9 (0.77 - 1.04)	0.15
AG	303 (13.8)	354 (15.6)			G	349 (0.08)	398 (0.09)		
GG	23 (1.1)	22 (1.0)							
rs7943404									
TT	734 (33.7)	763 (33.5)	0.46	0.44	T	2538 (0.58)	2618 (0.58)	0.97 (0.89 - 1.05)	0.44
CT	1070 (49.2)	1092 (48)			C	1812 (0.42)	1932 (0.42)		
CC	371 (17.1)	420 (18.5)							
rs11225395									
CC	628 (28.9)	735 (32.4)			C	2361 (0.54)	2566 (0.57)	1.09 (1.01 - 1.19)	**0.04**
CT	1105 (50.9)	1096 (48.3)	0.04	0.04	T	1983 (0.46)	1970 (0.43)		
TT	439 (20.2)	437 (19.3)							
rs1320632									
AA	1815 (83.6)	1894 (83.8)	0.44	0.64	A	3965 (0.91)	4140 (0.92)	1.04 (0.89 - 1.2)	0.64
AG	335 (15.4)	352 (15.6)			G	379 (0.09)	382 (0.08)		
GG	22 (1.0)	15 (0.7)							
rs1940475									
AA	528 (24.4)	620 (27.5)			A	2159 (0.50)	2322 (0.51)	1.07 (0.98 - 1.16)	0.13
AG	1103 (50.9)	1082 (48)	0.05	0.13	G	2171 (0.50)	2190 (0.49)		
GG	534 (24.7)	554 (24.6)							

rs1892886									
TT	1140 (52.9)	1274 (56.4)	0.04	0.01	T	3136 (0.73)	3395 (0.75)	1.13 (1.03 - 1.25)	**0.01**
AT	856 (39.7)	847 (37.5)			A	1178 (0.27)	1125 (0.25)		
AA	161 (7.5)	139 (6.2)							
rs17099436									
TT	1963 (89.8)	2017 (88.7)	0.34	0.18	T	4144 (0.95)	4283 (0.94)	0.88 (0.74 - 1.06)	0.18
AT	218 (10)	249 (10.9)			A	228 (0.05)	267 (0.06)		
AA	5 (0.2)	9 (0.4)							
rs2508383									
CC	1433 (65.9)	1436 (63.7)	0.28	0.18	C	3521 (0.81)	3601 (0.80)	0.93 (0.84 - 1.03)	0.18
CT	655 (30.1)	729 (32.3)			T	825 (0.19)	907 (0.20)		
TT	85 (3.9)	89 (3.9)							
rs1276284									
GG	886 (40.5)	1016 (44.9)	0.003	0.001	G	2774 (0.63)	3033 (0.67)	1.17 (1.07 - 1.27)	**0.001**
AG	1002 (45.9)	1001 (44.2)			A	1596 (0.36)	1497 (0.33)		
AA	297 (13.6)	248 (10.9)							

OR, odds ratio for minor allele, p*, p value for genotypic Pearson χ^2 test, P**, p value for trend test, P***, p value for allelic χ^2 test

3.2.5 MMP8 gene variation in the Polish breast cancer and melanoma study

All SNPs were in HWE. There were no statistically significant differences in allele or genotype frequencies detected, when analysed by conditional logistic regression, for MMP1 or MMP8 SNPs in the breast cancer part of the study. Similarly there were no significant differences detected for MMP1 SNPs in relation to the risk of malignant melanoma. Table 3.8 shows the MAF of the MMP1 SNPs and the MMP8 SNP rs11225395, in the Polish breast cancer and melanoma cases and their respective controls.

Table **3.8** MAF of the MMP1 SNPs and the MMP8 SNP rs11225395, in the cases and controls of the Polish study

MMP1 SNP	Location	MM cases (%)	MM controls	BC cases	BC controls
rs498186	Promoter	224/590 (0.38)	213/552 (0.386)	229/574 (0.399)	240/590 (0.407)
rs1144393	Promoter	252/592 (0.426)	235/554 (0.424)	249/584 (0.426)	248/612 (0.405)
rs475007	Promoter	251/586 (0.428)	249/558 (0.446)	256/598 (0.428)	268/616 (0.435)
rs514921	Promoter	144/588 (0.245)	129/552 (0.234)	153/592 (0.258)	145/616 (0.235)
rs494379	Promoter	124/592 (0.209)	127/542 (0.234)	108/586 (0.184)	145/602 (0.241)
rs3213460	Exon 1	78/588 (0.133)	72/556 (0.129)	95/584 (0.163)	89/608 (0.146)
rs470358	Intron 1	242/570 (0.425)	222/524 (0.424)	226/576 (0.392)	239/592 (0.404)
rs996999	Intron 4	132/590 (0.224)	121/544 (0.222)	122/582 (0.21)	132/598 (0.221)
rs5031036	Intron 5	45/580 (0.078)	47/526 (0.089)	43/588 (0.073)	51/602 (0.085)
rs491152	Intron 5	0/582 (0)	0/544 (0)	0/582 (0)	0/602 (0)
rs1051121	Exon 6	6/564 (0.011)	15/536 (0.028)	16/586 (0.027)	15/602 (0.025)
rs11225426	Intron 6	38/580 (0.066)	43/544 (0.079)	39/580 (0.067)	36/598 (0.06)
rs71250626	Intron 6	164/574 (0.286)	127/514 (0.247)	158/574 (0.275)	166/594 (0.279)
rs2071231	Intron 9	10/586 (0.017)	9/540 (0.017)	5/582 (0.009)	8/610 (0.013)
rs470215	Exon 10	208/586 (0.355)	184/538 (0.342)	207/584 (0.354)	201/602 (0.334)
rs2071230	Exon 10	41/570 (0.072)	40/536 (0.075)	36/558 (0.065)	39/590 (0.066)
rs7945189	3' region	62/584 (0.106)	51/544 (0.094)	53/580 (0.091)	70/610 (0.115)
rs1729376	3' region	37/588 (0.063)	40/554 (0.072)	41/592 (0.069)	42/608 (0.069)
MMP8 SNP					
rs11225395	Promoter	268/592 (0.453)	220/580 (0.379)	262/594 (0.441)	258/606 (0.426)

A positive association was found between MMP8 rs11225395 SNP and the occurrence of malignant melanoma. Conditional logistic regression analysis showed that the minor allele homozygote (T/T) was associated an increased risk of melanoma (OR 1.69, 95% CI 1.02 – 2.08; p = 0.040) with reference to the major homozygote (C/C); similarly the heterozygote genotype (C/T) also carried an increased risk of melanoma (OR 1.49 95% CI 1.03 – 2.17; p = 0.035) table 3.9. The frequency of risk allele was 0.453 in MM cases compared with 0.379 in controls (OR 1.54 95% CI 1.08 – 2.2; p = 0.017; Table 3.9).

Table **3.9** Genotype and allele frequencies of the MMP8 rs11225395 SNP Polish in melanoma cases and controls (conditional logistic regression)

	cases, n (%)	Controls, n (%)	OR (95% CI, P value)
C/C genotype	86 (29.1)	113 (39.0)	Reference
C/T genotype	152 (51.4)	134 (46.2)	1.49 (1.03 – 2.17, 0.035)
T/T genotype	58 (19.6)	43 (14.8)	1.69 (1.02 – 2.08, 0.040)
C allele	0.55	0.38	Reference
T allele	0.45	0.62	1.54 (1.08 – 2.2, 0.017)

CI, confidence interval; OR, odds ratio

Meta-analysis of rs11225395 SNP in association with breast cancer in the Strangeways and Polish studies

It was possible to perform a meta-analysis of the data for the rs11225395 SNP as this variant was tested in both the Strangeways and Polish case/control studies regarding breast cancer. The Strangeways study showed a significant association between rs11225395 and breast cancer, the Polish study did not. Meta-analysis of the two studies showed an association between the minor allele (T) and the occurrence of breast cancer

(OR 1.09, p = 0.03; both random and fixed effects). No significant heterogeneity was detected (Cochran's Q = 0.82, I^2 = 0).

3.2.6 Functional investigations into MMP8 and breast cancer

To test whether the rs11225395 (-799bp) promoter SNP has a direct functional effect on MMP8 expression in breast cancer cells luciferase reporter assays were carried out in the MDA-MB-231 breast cancer cell line. Experiments, in which luciferase reporter constructs incorporating MMP8 promoter segments containing either the C or T allele of the rs11225395 SNP were transfected into breast cancer cells, showed that the T allele had approximately 1.8-fold higher promoter activity than the C allele ($p = < 0.05$, Fig 3.6).

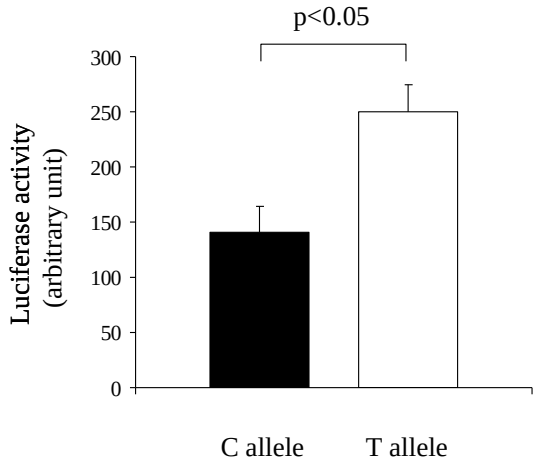

Figure 3.6. MMP8 gene promoter activity of the T and C alleles of the -799C>T SNP (rs11225395). Columns, relative MMP8 promoter activity of the T and C alleles in MDA-MB-231 breast cancer cells, measured by dual-luciferase assays as described in Materials and Methods. Data shown are mean (±SD) from three independent experiments.

3.2.7 Functional investigations into MMP8 and melanoma

To test whether the rs11225395 (-799bp) promoter SNP has a direct functional effect on MMP8 expression in melanoma cells luciferase reporter assays were carried out in the A2058 cell line. Experiments, in which luciferase reporter constructs incorporating MMP8 promoter segments containing either the T or C allele of the rs11225395 SNP were transfected into melanoma cells, showed that the T allele had approximately 2-fold higher promoter activity than the C allele (p = 0.015, Fig 3.7).

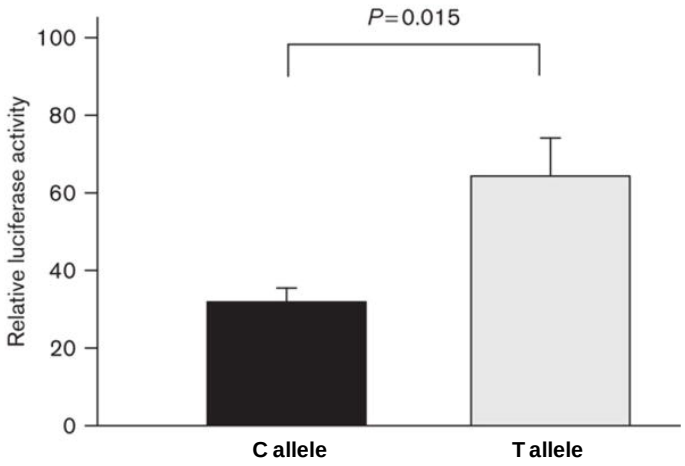

Figure 3.7. MMP8 gene promoter activity of the C and T alleles of the -799C>T SNP (rs11225395). Columns, relative MMP8 promoter activity of the C and T alleles in A2058 melanoma cells, measured by dual-luciferase assays as described in Materials and Methods. Data shown are mean (±SD) from three independent experiments.

3.3 Investigation into the role of MMP8 in atherosclerosis

3.3.1 MMP8 gene variation in relation to atherosclerosis

To study MMP8 gene variation in relation to atherosclerosis SNPs from the HapMap database were selected to capture/tag ($r^2 \geq 0.8$) common SNPs (with a minor allele frequency of ≥ 0.05) within the 5' upstream sequence, introns and 3' untranslated region. In addition to this the MMP8 gene proximal promoter (2 kb) and coding regions were resequenced. Examples of SNPs seen or identified by resequencing are exon 2 coding SNPs rs12803000 (synonymous) and rs1940475 (non-synonymous), and synonymous exon 6 SNP rs3740938 are shown in figure 3.8.

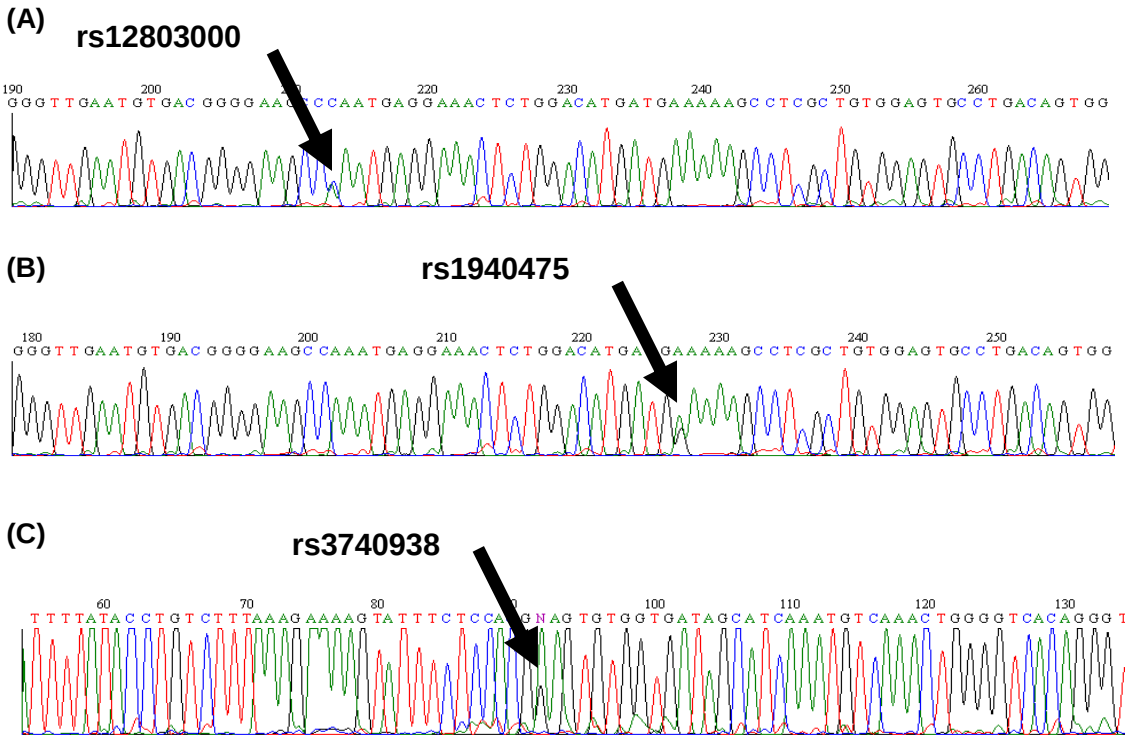

Figure 3.8. MMP8 SNPs identified by resequencing. (A) chromatogram of MMP8 exon 2 forward sequence segment showing rs12803000 A>C heterozygote, (B) chromatogram of MMP8 exon 2 forward sequence segment showing rs1940475 A>G (Lys>Glu) heterozygote, (C) chromatogram of MMP8 exon 6 reverse sequence segment showing rs3740938 G>A heterozygote.

Initially 1000 CAD patients from Southampton General Hospital were genotyped for the 16 SNPs that were identified with the combined strategies of resequencing and HapMap tagging; details of the SNPs are given in table 3.10.

Table 3.10: Human MMP8 gene SNPs examined in relation to atherosclerosis

dbSNP ID	Major allele	Minor allele	MAF in this study	MAF in HapMap	SNP location	Comment
rs10895353	A	G	0.10	0.07	Promoter, position -3345	tagging SNP
rs7943404	A	G	0.42	0.42	Promoter, position -2926	tagging SNP
rs11225395	C	T	0.43	0.48	Promoter, position -799	
rs1320632	A	G	0.09	0.11	Promoter, position -381	
rs2155052	C	G	0.10	0.10	Promoter, position -17	
rs17099450	G	C	0.05	0.04	Exon 1	non-synonymous (Cys3Ser)
rs3765620	A	G	0.43	0.48	Exon 1	non-synonymous (Ile32Thr)
rs12803000	T	G	0.05	0.04	Exon 2	Synonymous
rs1940475	C	T	0.49	0.52	Exon 2	tagging SNP, non-synonymous (Glu87Lys)
rs1892886	T	A	0.25	0.28	Intron 4	tagging SNP
rs17099436	T	A	0.05	0.06	Intron 4	tagging SNP
rs3740938	G	A	0.06	0.06	Exon 6	Synonymous
rs2508383	G	A	0.20	0.21	Intron 7	tagging SNP
rs1276282	C	T	0.09	-	3'-untranslated region	
rs1291327	G	A	0.10	-	3'-untranslated region	
rs1276284	G	A	0.34	0.38	3'-untranslated region	tagging SNP

The rs1940475 SNP was found to be associated with the extent of coronary atherosclerosis (p=0.01). The genotyping of an additional 1000 CAD patients from Southampton General Hospital for this SNP strengthened the association with the extent of coronary atherosclerosis, when analysed for the entire study population (n=2000, p=0.0008). The (T) allele had a lower frequency in patients with multi-vessel disease

than in patients with >50% stenosis in only 1 vessel (OR=0.80, 95% CI 0.70 – 0.91; Table 3.11). The above findings remain significant when taking into account multiple testing as calculated using the SNPSpD method. This method is less stringent the commonly used Bonferroni method; it does not assume all the SNPs are completely independent and utilises a spectral decomposition of matrices of pairwise LD between SNPs (183, 184, 200).

Table 3.11. Association between MMP8 gene variation and extent of coronary atherosclerosis in patients with coronary artery disease (n=2000)

rs1940475 SNP	Subjects with significant atherosclerosis* in one coronary artery	Subjects with significant atherosclerosis* in two or three coronary arteries	Odds ratio (95% confidence interval) † for significant atherosclerosis* in two or three coronary arteries
Genotype	n (%)	n (%)	
C/C	170 (22.2)	342 (27.7)	reference
C/T	398 (52.0)	632 (51.2)	0.77 (0.62-0.97), p=0.028
T/T	198 (25.8)	260 (21.1)	0.63 (0.49-0.83), p=0.0008
Total	766 (100.0)	1234 (100.0)	
Allele	frequency	frequency	
C	0.48	0.53	reference
T	0.52	0.47	0.80 (0.70-0.91), p=0.0008

*Significant atherosclerosis was defined by >50% stenosis
†calculated by logistic regression analysis with adjustment for age, gender, smoking, hypercholesterolemia, hypertension and diabetes.

The rs1940475 SNP associated with the extent of coronary atherosclerosis in CAD patients from Southampton was further evaluated in the Bruneck study, a population based prospective study. In agreement with the above study the (T) allele was found to be associated with a protective effect against carotid atherosclerosis over a 10 year follow up period; (OR 0.77, 95% CI 0.60 – 0.98) for atherosclerosis progression between 1990 and 1995; (OR 0.74, 95% CI 0.56 – 0.99) for atherosclerosis progression between 1995 and 2000 (table 3.12).

Table 3.12. Association between MMP8 gene variation and carotid atherosclerosis progression in the Bruneck study cohort (n=782)

Carotid atherosclerosis progression*	Odds ratio (95% confidence interval) ‡	Odds ratio (95% confidence interval)§
1990-1995	0.77 (0.60-0.98), p=0.032	0.73 (0.57-0.95), p=0.018
1995-2000	0.74 (0.56-0.99), p=0.040	0.78 (0.58-1.04), p=0.090
Change in carotid atherosclerosis score†	Regression coefficient (95% confidence interval)‖	Regression coefficient (95% confidence interval)#
1990-1995	-0.022 (-0.004 to -0.040), p=0.011	-0.025 (-0.042 to -0.008), p=0.003
1995-2000	-0.022 (-0.002 to -0.042), p=0.025	-0.022 (-0.042 to -0.002), p=0.032

*Carotid atherosclerosis progression was defined by the occurrence of atherosclerotic lesions in segments previously free of atherosclerosis or enlargement of non-stenotic lesions by a relative increase in the plaque diameter exceeding twice the measurement error of the carotid ultrasound examination method (158-161).

†Carotid atherosclerosis score, an indicator of atherosclerosis severity, was calculated by summing the maximum diameter of atherosclerotic plaques at 8 well-defined segments of the common and internal carotid arteries (158-161).

‡Odds ratio for carotid atherosclerosis progression related to each copy of the T allele, calculated by logistic regression analysis with adjustment for age, gender and baseline carotid atherosclerosis score.

§Odds ratio for carotid atherosclerosis progression related to each copy of the T allele, calculated by mutltivariate logistic regression analysis with adjustment for age, gender, baseline carotid atherosclerosis score, smoking, alcohol consumption, hypertension, diabetes, body mass index, waist-to-hip ratio, LDL and HDL levels, lipoprotein a, fibrinogen, the factor V Leiden mutation, C-reactive protein, ferritin and urinary albumin. Additional adjustment for soluble VCAM-1 yielded virtually identical results.

‖Regression coefficient calculated by linear regression analysis of $\log_e$-transformed carotid atherosclerosis score in relation to the T allele, with adjustment for age, gender and baseline carotid atherosclerosis score.

#Regression coefficient calculated by linear regression analysis of $\log_e$-transformed carotid atherosclerosis score in relation to the T allele, with adjustment for age, gender and baseline carotid atherosclerosis score and other variables as above.

Plasma levels of soluble VCAM-1 were found to be lower in individuals from the Bruneck study carrying the T allele of the rs1940475 SNP (p=0.014, table 3.13).

Table 3.13. Association between MMP8 genotype and plasma VCAM-1 level in the Bruneck study cohort (n=782)

MMP8 Genotype*	VCAM-1 (ng/ml)†	p value
C/C	661.2	0.014
C/T	624.5	
T/T	603.7	

*Genotype of the rs1940475 SNP; †Data shown are age- and sex-adjusted genometric means.

3.3.2 Functional investigations into MMP8 in atherosclerosis

3.3.2.1 Analysis of the effects of Ang 1 cleavage products by MMP8 on the murine VCAM-1 promoter via luciferase assay in endothelial cells

In endothelial cells transfected with luciferase reporter vectors containing a 263 bp sequence of the murine VCAM-1 promoter with either the NF-kB response element intact or ablated confirmed that the NF-kB element is functional. The promoter sequence in which the NF-kB element is ablated showed significantly less luciferase activity than the normal promoter sequence (untreated C166 cells p = 0.013, untreated bEnd.3 cells p = 0.039; fig 3.9 A and B respectively). ANOVA showed there was no significant difference of relative luciferase activity, for the intact VCAM-1 promoter, between any of the post transfection treatments (angiotensin I, MMP8, MMP8 and angiotensin I, or no treatment) in the C166 cell line or the bEnd.3 cell line (p = 0.574, p = 0.112; Fig 3.9 A and B respectively).

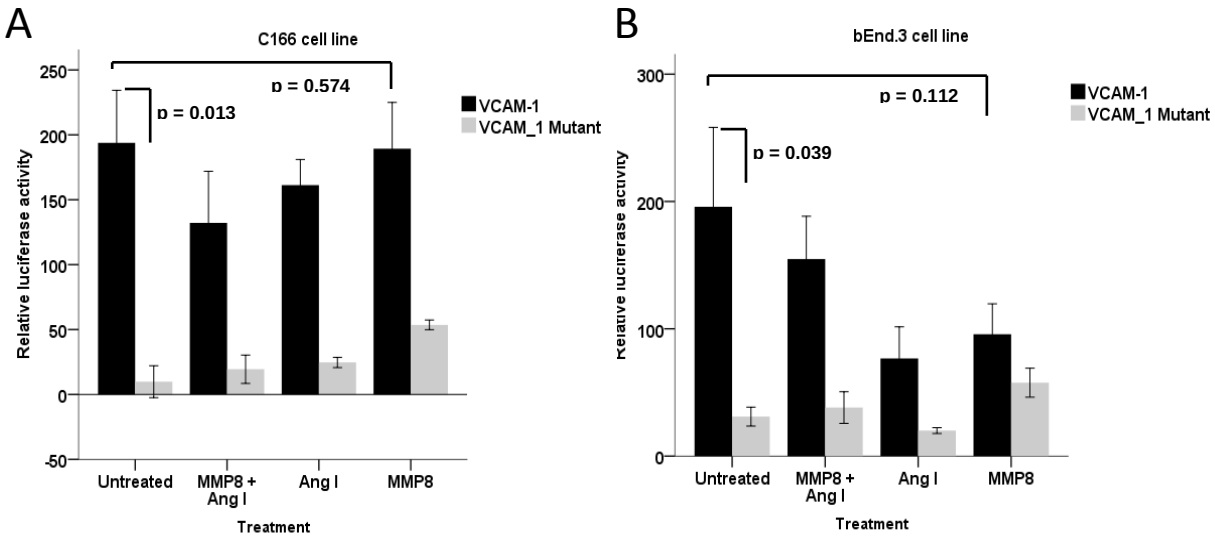

Figure 3.9. Relative promoter activity the VCAM-1 promoter with or without the NF-kB element ablated after post transfection treatments of angiotensin I, or MMP8, or MMP8 and angiotensin I, or no treatment; in (A) C166 cells, or (B) bEnd.3 cells. Activities were measured by dual luciferase assay described in the methods section. Representative of two independent experiments, each combination of treatment and plasmid was in triplicate. Error bars represent standard error of mean.

3.3.3 Biochemical and animal investigations of the role of MMP8 in atherosclerosis

3.3.3.1 Cleavage of Angiotensin I

A reported substrate of MMP8 is angiotensin I, which it converts into angiotensin II and Angiotensin 1-7 (194)). Angiotensin II is a potent vasoconstrictor and well established atherogenic molecule (201-203). To verify this MMP8 was incubated with angiotensin I, the cleavage products were then analysed by colleagues using liquid chromatography–electrospray tandem mass spectrometry. The results show that MMP8 converts Ang I principally into Ang II but also Ang 1-7 (fig 3.10)

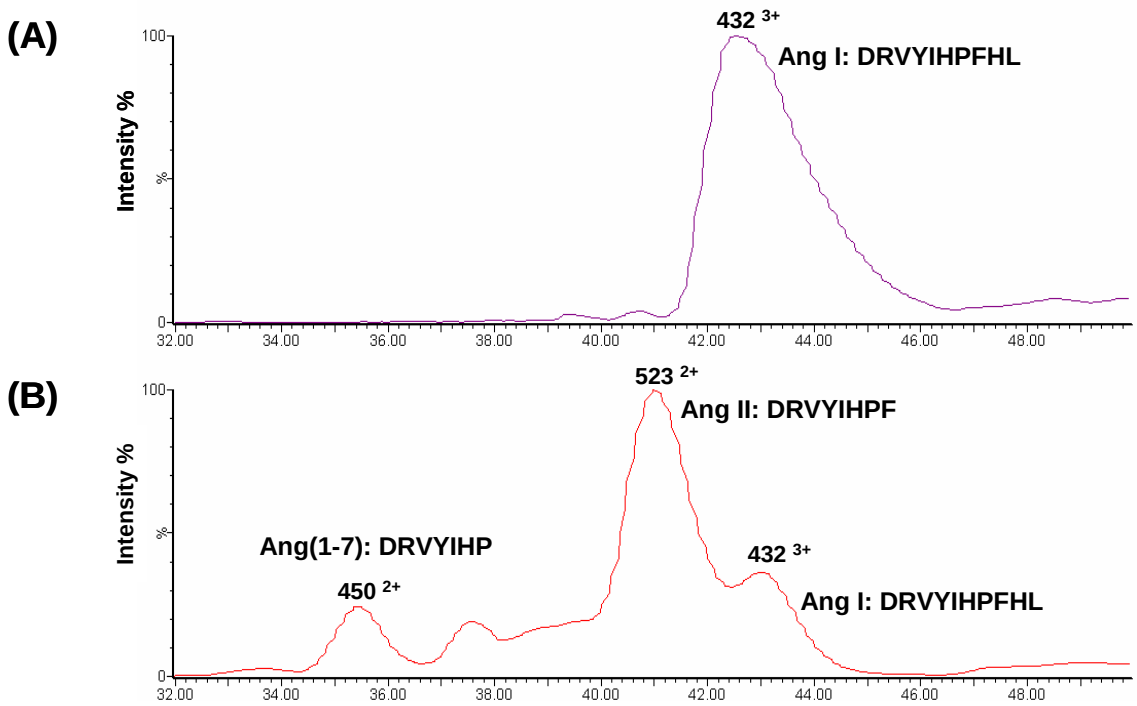

Figure 3.10. Cleavage of Ang I by MMP8, producing Ang II and Ang 1–7. **A**, Chromatogram of mass spectrometric analysis of uncleaved Ang I, showing the detection of Ang I peptide ion (*m*/*z* 432.84^{3+}). **B**, Chromatogram of mass spectrometry analysis of products of Ang I cleavage by MMP8, showing the presence of Ang II (*m*/*z* 523.79^{2+}) and Ang 1–7 (*m*/*z* 450.25^{2+}) peptide ions, in addition to Ang I (*m*/*z* 432.91^{3+}). The presence of Ang II, Ang 1–7, and Ang I in the respective peaks were confirmed by peptide fragmentation sequencing.

3.3.3.2. Reduced angiotensin II levels in double KO mice

To investigate whether the ability of MMP8 to process Ang I into Ang II could be reflected in the double knockout and control groups, plasma levels of Ang I and Ang II were measured. Ang II plasma levels were found to be lower in the double knockout group compared to the control group; contrastingly plasma Ang I levels, the precursor of Ang II, were found to be higher in the control group compared to the double knockout group (p=0.026 and p=0.018 respectively; fig 3.11 A and 3.11 B).

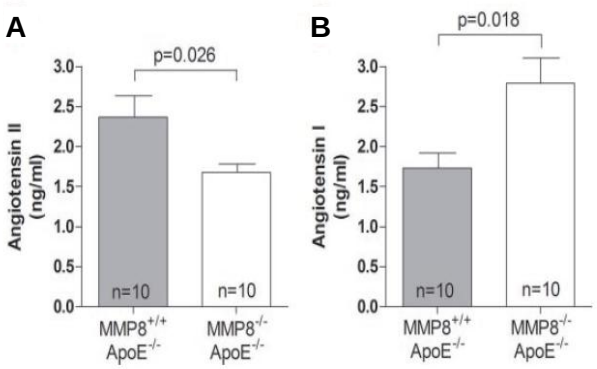

Figure 3.11. Reduced Ang II levels in MMP8-/-ApoE-/- mice. A and B, charts showing lower plasma Ang II level, but higher plasma Ang I level, in MMP8-/-ApoE-/- mice than in MMP8+/+ApoE-/- mice.

3.3.3.3 No change in other angiotensin forming enzymes

While it has been established that MMP8 can process Ang I into Ang II, other enzymes are capable of performing this role particularly ACE, chymase and cathepsins (194, 203); with this in mind the activity of ACE, and chymase and cathepsins was assessed to check for differences between the two groups. There was no significant difference between the 2 groups in the levels of ACE activity or the levels of chymase and cathepsins (p=0.921 and p=0.495 respectively; fig 3.12 A and 3.12 B).

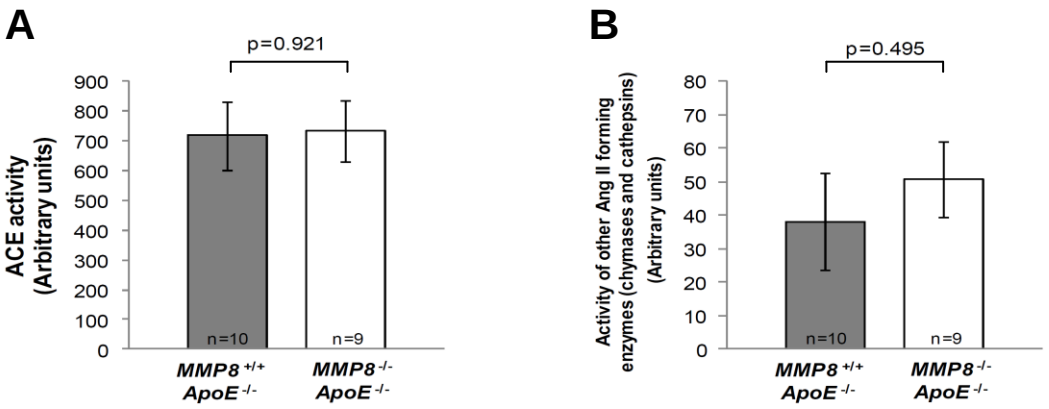

Figure 3.12. A and B, charts showing that there is no significant difference in the activity of other angiotensin II forming enzymes (A, ACE, B, chymases and cathepsins) between MMP8+/+/ApoE-/- and MMP8-/-/ApoE-/- mice. Data shown are mean ± standard error of mean.

3.3.3.4 Characterisation of atherosclerotic lesions

Results from the characterisation of atherosclerotic lesions of MMP8/ApoE double knockout mice (MMP8-/-ApoE-/-), fed a Western diet, and MMP8 wild type/ApoE knockout littermate controls (MMP8+/+ApoE-/-), also fed a Western diet, performed by colleagues are shown below.

MMP8/ApoE double knockout mice (MMP8-/-ApoE-/-) were generated, by crossing MMP8 knockout mice (122) with ApoE knockout mice (195, 196). MMP8-/-/ApoE-/- double knockout mice (n=10) and MMP8+/+ApoE-/- littermates (controls, n=10) were fed a Western high cholesterol diet for 12 weeks. Extensive aortic atherosclerotic lesions were observed in MMP8 wild type controls (Figure 3.13). The double knockout mice had substantially less aortic atherosclerosis (36% reduction compared with controls, p=0.007, Figure 3.13).

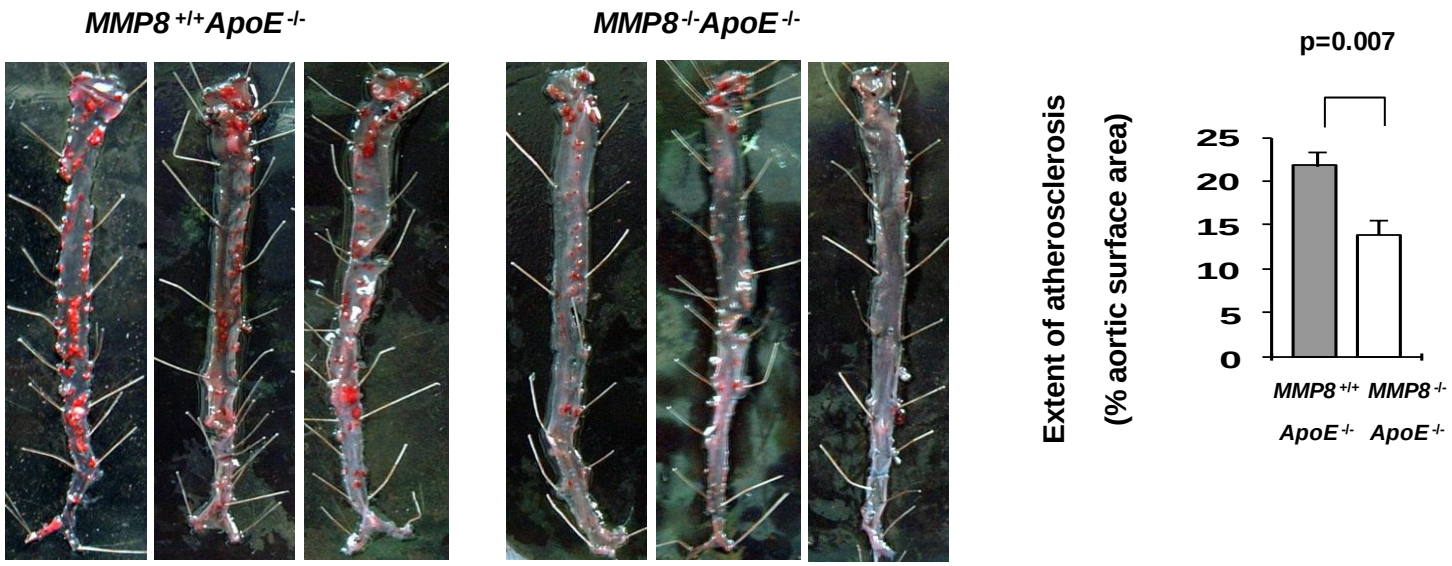

Figure 3.13. Reduced atherosclerosis in MMP8$^{-/-}$ApoE$^{-/-}$ mice. Panel A: representative images of Oil Red O stained en face preparations of aortas of MMP8$^{+/+}$/ApoE$^{-/-}$ (n = 10) and MMP8$^{-/-}$/ApoE$^{-/-}$ (n = 10) mice, and chart showing percentages of aortic surface area with atherosclerosis in the two groups of mice. Data presented are mean and standard error of mean.

3.3.3.5. Measurement of plasma triglyceride and cholesterol

Plasma levels of triglyceride were measured, there was no significant difference between the two groups (p=0.117; fig 3.14 A); neither was there a significant difference in plasma cholesterol levels (p=0.887; fig 3.14 B).

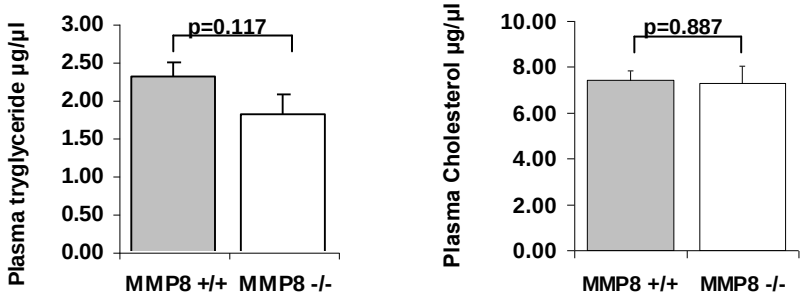

Figure 3.14. Plasma triglyceride and cholesterol in MMP8-/-ApoE-/- (n =10) and MMP8+/+ApoE-/- (n = 10) mice. Error bars represent standard error of mean.

3.3.3.6 Plasma VCAM-1 reduced in double knockout mice

Results from the animal studies agreed with those from the in vitro studies. Plasma sVCAM-1 levels, detected by ELISA, were found to be higher in MMP8+/+ApoE-/- mice than in MMP8-/-ApoE-/- mice (p=0.039; fig 3.15)

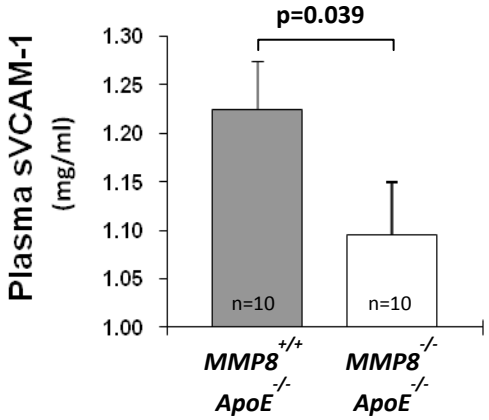

Figure 3.15 VCAM-1 expression in MMP8$^{-/-}$ApoE$^{-/-}$ mice compared MMP8$^{+/+}$ApoE$^{-/-}$ mice
Mean levels of soluble VCAM-1 in plasma. Error bars in the column are standard error of the mean

3.3.3.7. Measurement of plasma substance P and mesenteric substance P

Plasma levels of substance P were measured, there was no significant difference between the two groups (p=0.382; fig 3.16 A); neither was there a significant difference in levels of substance P in the mesentery (p=0.888; fig 3.16 B).

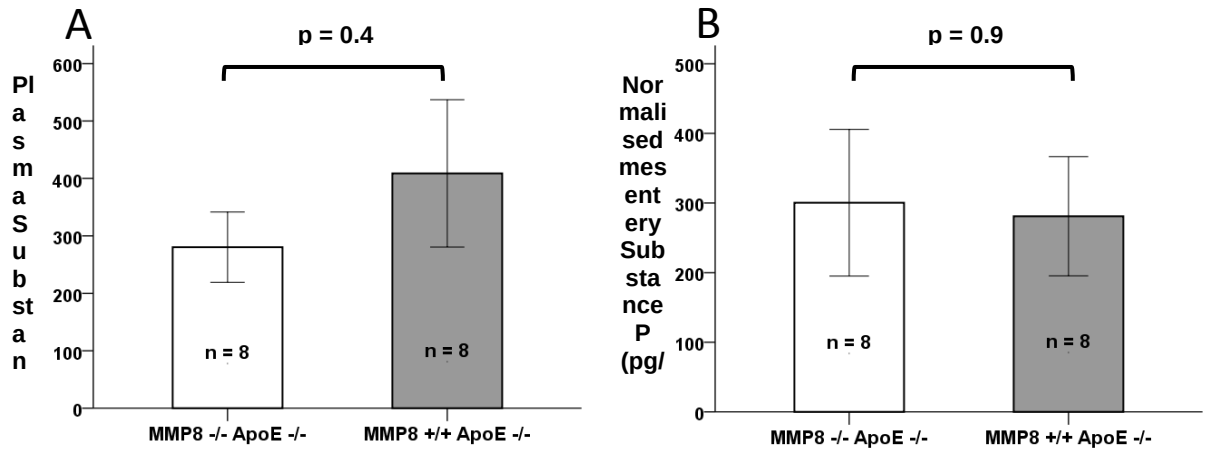

Figure 3.16. Substance P in MMP8$^{-/-}$ApoE$^{-/-}$ (n = 8) and MMP8$^{+/+}$ApoE$^{-/-}$ (n = 8) mice. (A) In plasma, (B) extracted from mesentery tissue (normalised by sample protein concentration. Error bars represent standard error of mean.

4.0 Discussion

4.1 Genetic investigations into MMP1, MMP8 and disease

4.1.1 Haplotype effects on MMP1 gene promoter activity in cancer cells

The results from the study of variation in the MMP1 promoter establish that there is a haplotypic effect exerted by multiple polymorphisms on promoter activity in different cancer cell lines in vitro; the effect is larger than that caused solely by the rs1799750 SNP; the 2G allele of which creates an Ets nuclear factor binding site leading to increased transcription (112). The results indicate that in most of the cell lines tested the rs1799750 SNP is the strongest individual driver of the haplotypic effect, but is not acting in isolation. In the melanoma cell line (A2058) all haplotypes containing the 2G allele of rs1799750 SNP had significantly higher promoter activity than those without. Evidence for the contribution of other SNPs towards overall haplotypic effects was given by the fact that there were significant differences between the promoter activities of the six 2G allele containing haplotypes in A2058 cells. Haplotype effects were also evident in the other melanoma cell line (A375), the breast cancer cell lines (MDA-MB-231 and MCF7), and a lung cancer cell line (A549). In addition to the '2G' containing haplotypes high expression was conferred by most frequent haplotype (G-A-T-G-A-T-T) in A375 and MD-MB-231 cells. The G-A-T-G-A-T-T haplotype was also amongst the higher expressing haplotypes in A549 cells; the GG-G-G-A-T-T-T haplotype, in these cells, had significantly higher expression than most of the other haplotypes. G-G-T-A-A-C-T and GG-G-G-A-T-T-T haplotypes produced significantly higher expression than the others in MCF7 breast cancer cells, the top expressing haplotype (G-G-T-A-A-

C-T) being only ‘1G’ one with high expression. Detection of differences in between haplotypic expression was possibly confounded in H69, HT-29, and SW-620 cells by the relatively high expression of the empty pGL3-basic vector; this was not observed in the A5028, A375, MDA-MB-231, MCF7, and A549 where significant differences were obtained. Previously undertaken investigations show that null vectors such as pGL3-basic can be trans-activated in some cell lines (204).

As stated, the investigation into MMP1 promoter activity indicates an effect caused by the rs1799750 (-1607) SNP in combination with one or more of the other SNPs studied. Previous studies have also shown haplotypic effects on the transcription of other genes; the cooperation of two or more of four promoter variants effects influence the transcriptional regulation of the cytokine IL6 (22); the interaction of three SNPs in the CETP gene promoter alter its activity and CETP plasma levels (23). Investigations either published concurrently or subsequently to the data presented here (193) continue to provide a strong case for the analysis of haplotypes, over that of single polymorphisms, in relation to gene activity and phenotype, with the discovery of haplotypes effecting O^6-alkylguanine-DNA alkyltransferase (MGMT) and lung cancer susceptibility (205), eye colour (206), and Janus kinase 2 (JAK2) associated diseases (207).

MMP gene regulation as covered in section 1.1.3.1 is partly dependent upon *cis*-elements within their promoter regions; the *cis*-elements are small DNA sequences recognised and bound to by transcription factor proteins. The binding sites may be created, augmented or lost dependent upon the allele present if a SNP occurs within a *cis*-element sequence, a pertinent example the Ets-1 binding site created by the

rs1799750 2G allele (112). Functional haplotype effects can occur when more than one SNP is present within a *cis*-element or there are SNPs in more than one *cis*-element sometimes changing the interaction of more than one transcription factor (TF). It follows that the ability of haplotypes to affect promoter activity can depend upon the complement of transcription factors active within a particular cell or cell line; different breast cancer cell lines have been shown to have varied TF profiles (208). The divergent effects of MMP1 promoter haplotypes seen in the different cancer cell lines, and between cell lines of the same type of cancer may be caused by a different TF profiles; the amount of influence on promoter activity by SNPs has been seen to vary dependent on cell type in other genes such IL6, MMP2 and MMP8 (22, 50, 209). In silico examination of the MMP1 promoter using the Genomatix SNPinspector program places the studied polymorphisms within putative TF binding sites (Table 3.3), this supports the notion that these SNPs may regulate transcription via TF binding but functional investigations are needed to verify any interactions. The haplotypic effect of the MMP1 promoter polymorphisms has already been established previously by colleagues, regarding risk of MI or promoter activity in THP1 cells (24); to date the majority of the genetic investigations into MMP1 and cancer have focussed solely upon the rs1799750 1G>2G SNP (25, 27, 29, 113-116, 210, 211). This analysis of the haplotype effects in cancer cell lines provides functional data that gives credence to the notion that genetic epidemiological studies would be better served genotyping multiple SNPs/haplotypes rather than just single SNP analysis; it shows that this method gives a better coverage of the genetic variations that confer differences in promoter activity when studying MMP1. The conclusion that use of haplotype analysis provides a greater amount of information than the single marker approach is not particularly surprising. Haplotype analysis has previously been well discussed by de Bakker *et al in* 2005 (168) with

reference to the statistical power of genotyping strategies, they also mention that the power to detect causal variants is decreased in areas of low LD as you are in effect testing fewer putative alleles, this is relevant in this case as this study shows that the LD between MMP1 promoter SNPs is considerably low compared to some other genes e.g. MMP3 (212), probably due to recombination hotspot around the MMP1 SNPs (Fig 3.2). Recent studies into cutaneous malignant melanoma also support the notion that these seven polymorphisms and their haplotypes can influence risk of melanoma (213) and progression of melanoma (214).

To summarise, this multi-marker analysis of MMP1 gene promoter SNPs shows that the MMP1 promoter polymorphisms exert haplotype effects on MMP1 promoter activity in cancer cells. The results show the advantage of examining haplotypes over single marker studies, in genetic investigations of the MMP1 gene and cancers.

4.1.2 MMP1 gene variation in malignant melanoma and breast cancer

Case control analysis of MMP1 gene SNPs and melanoma or breast cancer failed to find any significant association in the Polish study participants. This was despite the fact that the study encompassed five of the SNPs (rs498186, rs1144393, rs475007, rs514921, and rs494379) that were involved in the functional analysis of the promoter carried out earlier, shown to exert a haplotypic effect in A2058 and A375 melanoma cells lines, and MCF7 and MDA-MB-231 breast cancer cell lines. As always with this type of investigation, a small sample size and relatively low power means it is more difficult to uncover small or moderate effects and caveats should be provided for the negative interpretation. The negative result not preclude associations being found in larger

studies, indeed a larger Texan melanoma study found a significant association with three of the above SNPs (rs1144393, rs475007, and rs494379) and susceptibility after adjustment for age, sex, family history and sun-exposure-related risk factors (213). The same group later found associations with MMP1 promoter SNPs and clinicopathological factors involved in melanoma progression (214).

4.1.3 MMP8 gene variation and breast cancer

The investigations on MMP8 gene variation in relation to breast cancer initially found an association with lymph node metastasis and four SNPs, rs11225395, rs1940475, rs1892886, and rs1276284, in patients in the Leuven breast cancer study. The most interesting discovery of reduced risk of lymph node metastasis conferred by the minor allele (T) of the rs11225395 SNP; a polymorphism purported to have an effect on MMP8 gene expression in earlier studies (50). This finding was supported by collaborators with further analysis of this SNP in patients from the Shanghai breast cancer study (Section: A4.1). They found the T allele to be associated with reduced cancer relapse and increased survival. Functional analysis of this polymorphism using luciferase reporter assays with the MDA-MB-231 breast cancer cell line showed the T allele to have higher promoter activity than that of the C allele. Further to this electrophoretic mobility shift experiments upon MDA-MB-231 nuclear extract showed protein interaction with the T allele and none with the C allele; suggesting a direct functional role for the rs11225395 SNP (Section A4.2). The above findings support the case for an inhibitory effect of MMP8 on breast cancer metastasis as put forward by Montel *et al*. (121), and Argarwal *et al*. (120).

A well established prognostic marker of breast cancer, lymph node status is probably the strongest independent predictor of disease free survival and overall survival (215); lymph node involvement at the primary diagnosis predicts a poor outcome after the first relapse independent of disease free survival or location (216). Often the first site of metastasis in breast cancer, the involvement of lymph nodes indicates that the associated primary tumour is of an aggressive metastatic phenotype (154, 216). In the study presented here associations between lymph node metastasis and MMP8 SNPs were found, the strongest being with rs11225395. Additionally collaborators in an independent study observed that the less frequent T allele was associated with reduced cancer relapse and improved survival after breast cancer diagnosis in Chinese women (Tables A1 & A2, respectively). Although a trend was seen in all cases a significant association between the T allele and better prognosis was shown in Chinese women with early stage cancer (TMN stage 0-II) (Tables A1 & A2, Figure A2; see p174-176); the classification given when there is no involvement of other tissues or organs, any spread of cancer cells is restricted to lymph nodes only. These findings are in agreement with previous murine studies lymph node metastasis was much increased when MMP8 was knocked down in animals with breast cancer cells orthoptically implanted, a much smaller effect was seen with metastasis to the lung, therefore it was posited that MMP8 offers some protection against invasion via the lymphatic system, and less against invasion via the blood circulatory system (121). Also in agreement is a study that is subsequent to the publication of the data presented here (48); it was found that expression of MMP8 in humans correlated with a lower incidence of lymph metastasis and a better prognosis (217).

Luciferase reporter assays of rs11225395 revealed that the minor T allele had greater expression in breast cancer cells than the C allele. Additionally when the two alleles were subjected to electrophoretic mobility shift assay, no DNA-protein complexes were detected in conjunction with C allele. However three such complexes where seen with the T allele (Figure A3; see p180); the DNA-protein complexes may have been different nuclear proteins or three different fractions of the same protein. It is possible that the increased expression seen with the minor T allele is due to the nuclear proteins selectively binding to it acting as transcriptional enhancers. There were no good matches for consensus *cis*-elements with the rs11225395 T allele sequence when querying transcription factor binding databases but these are not yet exhaustive and so the nuclear protein may not be present within the databases.

Collaborators in Shanghai examined only the rs11225395 SNP because within the Leuven study it was most significantly associated with lymph node metastasis; in LD with the other three SNPs associated with metastasis (i.e. rs1940475, rs1892886, and rs1276284; in Caucasian & Chinese populations) and had been suggested to have an effect on MMP8 gene expression in the literature (50). Also haplotype analysis of the nine SNPs in the Leuven study showed that the T allele of rs11225395 tagged the haplotypes associated with reduced lymph node metastasis (HAP 2, and HAP 4) whilst the C allele tagged haplotypes associated with higher metastasis risk (HAP 1, and HAP 3).

Whilst the minor alleles of rs11225395, rs1940475, rs1892886, and rs1276284 were associated with a reduced risk of lymph node metastasis; not all of the respective alleles need have a direct functional effect on the activity of the MMP8 promoter or enzyme,

associations may be indicative of LD between the alleles and true functional variants. The functional studies of the rs11225395 SNP presented in sections 3.2.5 & A4.2 evidence a direct functional role for this promoter SNP aligning with the minor allele's association with reduced risk of lymph node metastasis and cancer relapse and improved survival. The rs1940475 variant is a plausible candidate for a functional role as it is a non-synonymous SNP occurring in exon 2 of the MMP8 gene. The SNP results in a glutamate to lysine substitution at amino acid residue 87 which is proximal to the cysteine residue that forms part of the 'cysteine switch' motif and mechanism involved in conversion of the zymogen into the active enzyme. While at the time that the data discussed here was published (48) there had been no functional data for this SNP, subsequent functional analysis has shown the T allele to be less amenable to activation (Section A4.3.1.) and will be discussed in relation to atherosclerosis further on. The other two SNPs found to be associated with lymph node involvement, rs1892886 and rs1276284, are located in intron 4 and the 3' flanking region of the gene respectively, hence they are less likely candidates for functional role. Currently there is no functional data for these polymorphisms; none of the data, functional or otherwise precludes any or all of the four SNPs from tagging the effects SNPs not tested in this study.

As mentioned in section 1.2.5 other MMP SNPS have been associated with cancer; Table 1.1 shows that most of the alleles leading to increased expression of the relevant MMP are associated with an increased susceptibility to cancer and/or metastasis; the data presented here shows a contrasting anti-tumourigenic effect of the higher expressing T allele of the rs11225395 MMP8 SNP; MMP12 is the best example of another MMP found to be beneficial by genetic studies in cancer; the lower expressing allele of rs2276109 is associated with poor prognosis in bladder cancer (55).

The small sample size of Leuven study gives some limitation to definitive conclusions regarding MMP8 variation and breast cancer, although this was somewhat remedied by the complementary findings by collaborators in a larger independent study with strong methodology (The Shanghai Breast Cancer Study). In common with all research any conclusions drawn can only be verified by replication and further work.

Further investigations into MMP8 gene variation and breast cancer encompassed the genotyping of the 'Strangeways' breast cancer cases (SEARCH) and controls (EPIC) (156), and genotyping cases and controls from a Polish study (162). This revealed that three of the four SNPs associated with lymph node metastasis in the Leuven study were significantly associated with increased susceptibility to breast cancer in the 'Strangeways' population. The SNPs being rs11225395, rs1892886, and rs1276284, the minor alleles of each polymorphism were found to have higher frequencies in patients with breast cancer compared to controls. The promoter SNP rs11225395 was the only MMP8 variant to be investigated regarding breast cancer in the Polish study; unlike the Leuven and Strangeways studies, no significant association was found. Since the both the Strangeways study and the Polish study are case control studies of breast cancer involving Caucasians of European descent it was feasible to perform a meta-analysis combining the results for the rs11225395 SNP in the two studies. The meta-analysis showed an association between the minor allele (T) and the occurrence of breast cancer with no significant heterogeneity was detected.

Despite significant associations involving the same SNPs in three studies, these results indicate diversity in the findings rather than replication and complementation. This is

best illustrated by focus on just the associations attributed to the minor (T) allele of rs11225395; in the Leuven study this allele confers a beneficial reduced risk of lymph node metastasis. The beneficial effect is supported by independent findings by collaborators, albeit in a non-Caucasian population (Chinese women). Contrary to these beneficial associations, the results from the Strangeways study and its meta-analysis with the Polish study indicate an increased risk of breast cancer conferred by the T allele. While Polish study is of a relatively small size, the Strangeways study consists of 4550 individuals giving it a reasonable power to detect causal variants. It must be pointed out that, while apparently divergent, the end points are not the same pertaining to different stages of a complex disease (initiation & progression). Furthermore only patients were genotyped in the Leuven and Shanghai studies whereas the Polish and Strangeways study involved both patients and healthy controls. Possible explanations for this dichotomy are type 1 error, or even a potential 'flip-flop' effect (218) caused by the level of correlation with a true causal variant at another locus differing in different populations. However also presented here is reasonable evidence for the rs11225395 polymorphism to be a functional SNP; luciferase assays in both the MDA-MB-231 and A2058 cancer cell lines, and EMSA results from MDA-MB-231 nuclear extract. Recent investigations into cardiovascular disease also show the T allele significantly associated with higher levels of serum MMP8 (219). The majority of published evidence regarding MMP8 and breast cancer points to a protective function (120, 121, 217); regarding other neoplastic pathologies there is also evidence to show a protective role against lung cancer (49). Mutational and cell analyses of MMP8 suggest a tumour suppressor function in melanoma (220). Finally, subsequent work by the Shanghai breast cancer study was able to replicate the initial finding (published in conjunction with the Leuven study) (48); genotyping an additional 5192 breast cancer patients; women homozygous

for the TT genotype had better overall survival than women with the CC genotype (HR: 0.6, 95% CI: 0.4–0.9, p = 0.02) (221).

The ‘flip-flop’ between the Leuven and Strangeways studies was not limited to the rs11225395 SNP but included rs1892886, and rs1276284. Taken at face value these data demonstrate a complex relationship between MMP8 and breast cancer that may involve the promotion of breast cancer initiation but thereafter providing a protective role regarding metastasis lymph node metastasis and prognosis. A similar conclusion was drawn for an allele of promoter polymorphism of the chemokine RANTES “a risk factor for HIV transmission and as a protective factor for HIV progression” (222). Our findings combined with both prior and post studies build a strong argument the importance of MMP8 in cancers, particularly in a protective role.

4.1.3 MMP8 gene variation and malignant melanoma

Similar to the rs11225395 breast cancer results from the Strangeways study and its meta-analysis with the Polish study; the T allele of this MMP8 promoter SNP was significantly associated with an increased risk of malignant melanoma. Luciferase reporter assays show this allele has approximately a twofold higher activity than the major (C) allele in melanoma cells (A2058). Current literature favours a tumour suppressor role for MMP8 in melanoma; Palavalli *et al* (220) found it to be frequently mutated in melanoma, the wild type was able to inhibit human melanoma cell growth in vitro and suppress metastasis in vivo. A separate study showed that overexpression of MMP8 causes inhibition of invasion and transendothelial migration of murine melanoma cells in vitro, and is anti-metastatic in vivo (217). However the genotyping the exon 2 non-synonymous SNP (rs1940475) by different case control study revealed

no association with melanoma (223). Only one other study shows a potential negative role for this enzyme regarding melanoma; it found higher MMP8 serum levels to associated with melanoma ulceration vascular invasion (224).

The results presented here indicate, as potentially with breast cancer, that MMP8 may increase susceptibly to this disease but play a suppressing role further down the line.

4.1.4 MMP8 gene variation and atherosclerosis

The study into MMP8 gene variation in CAD patients found association with the extent of coronary atherosclerosis. The same missense variation was also associated with the progression of carotid atherosclerosis and circulating levels of soluble VCAM-1 in the prospective Bruneck study. The rs1940475 SNP is situated in the pro-domain of MMP8, proximal to residues involved in the cysteine switch activation mechanism. Western blot analysis (Section A4.3) showed the missense allele had an effect of reduced MMP8 zymogen activation (Figure A4).

Similar to investigations into cancer, most early efforts to elucidate the role of MMPs in cardiovascular diseases concentrated on their matrix remodelling properties and ability to degrade structural components of the ECM. Undoubtedly this aspect of MMP functionality is involved in part in the development and even acute episodes of CVD (69). It is emerging however that this is not the whole story, more recently other studies have begun to investigate various effects that MMPs can have through the processing of non-matrix molecules such as cell bound growth factors and receptors, adhesion molecules, cytokines and other MMP zymogens (6). The link seen in this study between

MMP8 gene variation and the extent of atherosclerosis and circulating levels of soluble VCAM-1 points to another potential route by which MMP8 can be involved in the pathogenesis of atherosclerosis. VCAM-1 is not the only atherogenic molecule that can be linked to MMP8, as it is able to generate Ang II from Ang I (194), The generation of Ang II by MMP8 provides a plausible mechanism for part of MMP8's role in atherosclerosis. Potential Ang II mediated pro-atherogenic effects include elevated blood pressure, production of ROS, activation of NF-kB and subsequently induced molecules that not only include VCAM-1, but also MCP-1 and ICAM-1 (225). Induction of adhesion factors such as VCAM-1 and ICAM-1 facilitate the recruitment of monocytes which are then attracted into lesions via MCP-1 chemokine gradients. Ang II has also been shown to induce ox-LDL-receptor LOX-1, known to play a prominent role in the activation of the endothelium (226). Unfortunately it was not possible to query the relative levels of Ang I or Ang II in the Southampton CAD patients or the Bruneck Study, in order to detect a potential link with MMP8 gene variation and these factors. Further exploration of a mechanism for the involvement of MMP8 with the pathogenesis of atherosclerosis is discussed in the section concerning biochemical and in vivo animal studies.

4.2 Effects of Ang I cleavage products by MMP8 on the murine VCAM-1 promoter

Luciferase assays used to assess effects of Ang I cleavage by MMP8 on the murine VCAM-1 promoter confirmed that it has a functioning NF-kB response element; this is not in itself surprising as the human equivalent has previously been confirmed (185), added to this is that the murine and human genes share considerable homology (186). It was surprising that these assays failed to show significant differences in the effects of the aforementioned cleavage products on the promoter activity (see Fig 3.9);

particularly as Ang II is known to upregulate VCAM-1 (227), and as flow cytometry analysis performed concurrently by a colleague showed an increased VCAM-1 expression. A relatively small part of the proximal VCAM-1 promoter was assessed; it is possible that distal *cis*-elements for other transcription factors were required to work in conjunction NF-kB. Ultimately the results from the flow cytometry provided a closer model to in vivo physiological situations; as such this avenue was not pursued further.

4.3 Information from biochemical and animal studies of MMP8 and atherosclerosis

The following section discusses work in this study that was largely collaborative; as such a substantial proportion of the results were not directly generated by this investigator. Brief descriptions of the methods, along with those results are presented in the appendices (specifically Section A4.4 onwards). They are provided and discussed because they give context the data presented here and give a fuller story of the potential mechanism of MMP8 in the pathogenesis of atherosclerosis.

As mentioned MMP8 has the ability to process Ang I into Ang II , the principal effector molecule in the Renin-Angiotensin system (RAS) (225), a finding confirmed in this study. It was also found that products of Ang I cleavage by MMP8 induced elevated expression of VCAM-1 in murine endothelial cells in vitro (Figure A6), previously shown to be induced by Ang II (227, 228).

Prior to this study the loss of MMP8 had not been characterised using the ApoE deficient murine atherosclerosis model (195, 196). Here it was shown that the extent of the disease, induced by a high fat western diet, was much reduced in animals also deficient in MMP8 when compared with animals with the functional MMP8 wildtype.

There was a reduced amount of oil red O staining and fewer inflammatory cells were seen in the lesions of the MMP8 deficient mice, which also had higher collagen content (Figure A8). In agreement with biochemical data on the generation of Ang II by MMP8 there were lower levels of the vasoactive, pro-atherogenic molecule Ang II were seen in the double knock-out animals with concurrent higher levels of the precursor molecule Ang I. Consistent with the relative levels of Ang II in the two groups, blood pressure was found to be lower in the MMP8 deficient mice than the controls (Figure A9). This was in agreement with the finding that there were lower levels of VCAM-1 expression observed in the MMP8 KO mice compared with the controls (Figure A10). This correlates very well with the data provided by colleagues showing reduced leukocyte recruitment/rolling in the MMP8 deficient group of mice (Figure A11).

The principle enzyme associated with the conversion of Ang I into Ang II is ACE, consequently many anti-hypertension therapeutics have been ACE inhibitors. ACE like MMP8 is a zinc dependent protease and belongs to the metallopeptidase superfamily (229, 230). An inherent feature of this superfamily is the considerable conservation seen in the sequence and structure of their catalytic domains (231), illustrated by coincident substrates and also the inhibition of MMPs by ACE inhibitors captopril, enalapril, lisinopril, and quinapril (232-237). Apart from ACE and MMP8, some cysteine and serine proteases have been shown to have the ability to perform the Ang I to Ang II conversion, these include chymases, and cathepsins (203). There were no significant differences in the levels of activity of ACE, chymases or cathepsins between the two groups of animals, enhancing the probability that MMP8 can cause RAS mediated effects, particularly in physiological or pathophysiological situations with a scarcity of the other enzymes.

Another potential avenue by which MMP8 may influence the progression of atherosclerosis is the inactivation of substance P (194). The MMP8 enzyme is able to cleave between C terminus residues: Gly[9] and Leu[10], without the C-terminus residues substance P is unable to bind to tachykinin receptors (238). Similar to cleaving Ang I, the degradation of substance P by MMP8 can potentially lead to elevated blood pressure as it is generally a potent vasodilator, although when acting upon neurokinin-1 receptors on SMCs substance P can have vasoconstrictor properties (239). Relevantly substance P may activate mast cells and induce intraplaque haemorrhage (240, 241). Measurement of plasma and mesenteric substance P did not show any significant differences between the two groups making it less likely that this in an avenue by which MMP8 can affect atherosclerosis.

There have been several previous studies using murine atherosclerosis models showing the individual roles of MMPs (shown in Table 1.6) are complex, some appearing play a protective role while others appear to further the extent of disease. In an MMP1 mouse model, human MMP1 (hMMP1) was expressed in macrophages, as there is no murine homolog. The study found that lesion was size reduced in the hMMP1 transgenic animals but they also reduced collagen content, although there was no sign of plaque rupture (242). The third collagenase (MMP13) was seen to have a role in plaque collagen catabolism, knockout mice having increased content of interstitial collagen in their lesions (243). MMP2 (gelatinase-A) deficient mice were found to have reduced plaque and decreased SMCs, with no upregulation of MMP9 (gelatinase-B) (244). Conclusions on the role of MMP9 are not as clear; some studies indicate a detrimental effect, knockout animals having impaired SMC migration and smaller aortic lesion size

(137, 245, 246). Additionally an activated version of MMP9 when expressed from macrophages induced considerable plaque disruption (247). However another murine study focussing on lesions in the brachiocephalic artery showed that animals deficient in MMP9 had lesions that were much increased in size with an unstable phenotype. The same study concurrently investigating MMP12, showed that there were smaller lesions and a more stable phenotype in the double knockout group (142). Murine studies with MMP3 suggest a protective effect, enzyme deficiency causing increased lesion size and reduced SMC content (142, 248). MMP7 deficiency seemed to have a relatively neutral effect not causing any significant change in lesion development or stability, although lesions did show increased SMC compared to littermates only deficient for ApoE (142). MMP14 deficiency is lethal by 3 weeks, as such a study gave irradiated LDLR deficient mice either $MMP14^{-/-}$ or $MMP14^{+/+}$ bone marrow derived cells (BMDCs). There was more interstitial collagen within the lesions of the animals with $MMP14^{-/-}$ BMDCs, although no other change in size or content (249). Along with MMP8 several MMP's (MMP1, MMP3, MMP13 and MMP14) were shown to reduce the collagen content of lesions, potentially reducing plaque stability. However, in these models, MMP8 is unique correlated to decreased collagen, more inflammatory cells, and larger lesions.

This part of this study with regard to MMP8 and atherosclerosis has some limitations. Only the aorta was studied as a site of lesion formation, Johnson and colleagues showed studying the brachiocephalic site can sometimes give different results (142). The data was collected for only the 12 week time point on a western diet. It would be interesting to compare shorter and longer time points in order to gain more information to its role in the initiation and progression of the disease. Furthermore it would have been advantageous to investigate for evidence of plaque rupture (250). The animal study has

highlighted a potential route for MMP8 to affect atherosclerosis via the conversion from angiotensin I to angiotensin II. It would have been pertinent to investigate any correlation between MMP8 genotype and Ang II levels in the human studies (Southampton CAD patients and Bruneck), unfortunately these data are unavailable.

In summary both human and murine studies pointed to an important role for MMP8 in atherosclerosis. In combination with prior associations between its plaque levels and unstable phenotype (149-151) there is a compelling case for a significant role for MMP8 in this disease.

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Appendices

Appendix 1: Reagents, buffers and solutions

500 ml 10X ELB (erythrocyte lysis buffer) - 1X is to be autoclaved

5.05g	Potassium bicarbonate
41g	Ammonium chloride
2 ml	0.5M EDTA (pH 8)
To volume	H_2O

500 ml NLB (nucleic lysis buffer)

5 ml	1M Tris (pH 8)
40 ml	5M NaCl
2 ml	0.5M EDTA (pH 8)
To volume	H_2O

1 L 50X TAE (Tris-acetate-EDTA)

242g	Tris base
57.1 ml	Glacial acetic acid
100 ml	0.5M EDTA (pH 8)
To volume	H_2O

1 L 5X TBE (Tris-borate-EDTA)

54g	Tris base
27.5g	Boric acid
20 ml	0.5M EDTA (pH 8)
To volume	H_2O

LB (Luria-Bertani) medium

1%	Tryptone
0.5%	Yeast extract
1%	NaCl
97.5%	H_2O

LB (Luria-Bertani) agar

1%	Tryptone
0.5%	Yeast extract
1%	NaCl
1.5%	Agar
96%	H_2O

Appendix 2: MMP1 amplification primers and enzymes used for restriction fragment length polymorphism analysis

Genotyping

-1607 GG>G (rs1799750) TCGTGAGAATGTCTTCCCATT (PCR forward primer)

TCTTGGATTGATTTGAGATAAGT**G**A**A**ATC (PCR reverse primer, mismatched nucleotide in bold)

-839 G>A (rs473509) CCCATGATAATGATGGGCAAGGGGTGGG (PCR forward primer)

GCT**T**GAGTGCAGCGGCACAATCACAGCT (PCR reverse primer, mismatched nucleotide in bold)

-755 G>T (rs498186) TGATCCTCCCACCTCAGCCTCTTC**C**G (PCR forward primer, mismatched nucleotide in bold)

CCCAGAATTTCAGGAGGCCGAGGCAG (PCR reverse primer)

-519 A>G (rs1144393) TATAGGCTTGAGCCACCATG (PCR forward primer)

CACAGGTCAAAGAGTACTCC (PCR reverse primer)

-422 T>A (rs475007) TATAGGCTTGAGCCACCATG (PCR forward primer)

CACAGGTCAAAGAGTACTCC (PCR reverse primer)

-340 T>C (rs514921) AAAAAGACTGGTTCTGATGGTC**T**TAA (PCR forward primer, mismatched nucleotide in bold)

GAACTTCAGTCAGTACAGGTGCC (PCR reverse primer)

-320 T>C (rs494379) TTATGACCATCAGAACCGG (PCR forward primer, mismatched nucleotide in bold)

CACACTCTGCCATGTAAACAGTGG (PCR reverse primer)

Restriction endonuclease that specifically cleaved 1 of the 2 alleles. The restriction endonucleases used were XmnI (for -1607GG>G), HindIII (for -839G>A), MspI (for -755G>T), KpnI (for -519A>G), BanI (for -422T>A), AflII (for -340T>C), and HaeIII (for -320T>C)

Appendix 3: MMP8 amplification and sequencing primers

Exon1F:	5’ GTTGCTTCATATTCCTGATGC 3’
Exon1R	5’ AACAAGAGAAAACAACCCACA 3’
Exon2F:	5’ GGGTTGCAAAATGACCAGA 3’
Exon2R:	5’ TCCCAAGAACAGTGTCTGTCAT 3’
Exons3_4F:	5’ GGTAGCACACCATGATGCAA 3’
Exons3_4R:	5’ CAAATGATCACTTTTTGTGAGTTTG 3’
Exon5F:	5’ CAGCTTTTCTGGGTTCTAAGA 3’
Exon5R:	5’ GGGAGTGTTCGCCTATTC 3’
Exon6F:	5’ TGAGAAGTCTCTGATGTACCCAAG 3’
Exon6R:	5’ GGAAAAGGTGACTAACGTGTGA 3’
Exon7F:	5’ CTCCCAGCAATCCTACAAGG 3’
Exon7R:	5’ GGCCCAACCCTAGTCTTCTG 3’
Exon8F:	5’ TGTGCAAGACCAAGAAGTGG 3’
Exon8R:	5’ TCAAAAGCCATGGTAACATCA 3’
Exon9F:	5’ TTGGTGTTAAATCGGATAAC 3’
Exon9R:	5’ TTGACACTAACCTGGTCAGCA 3’
Exon10F:	5’ TGGGATTACAGGCATTAGCC 3’
Exon10R:	5’ GCAGGACACAATGTAACGAAAA 3’

Appendix 4: Supporting data from collaborators/colleagues

A4.1 The Shanghai Breast Cancer Study

A cohort of 1455 Chinese breast cancer patients recruited between 1996 and 1998 as part of a population based case control study, the Shanghai Breast Cancer Study (165, 251), there was a median follow up time of 7.1 years (252).

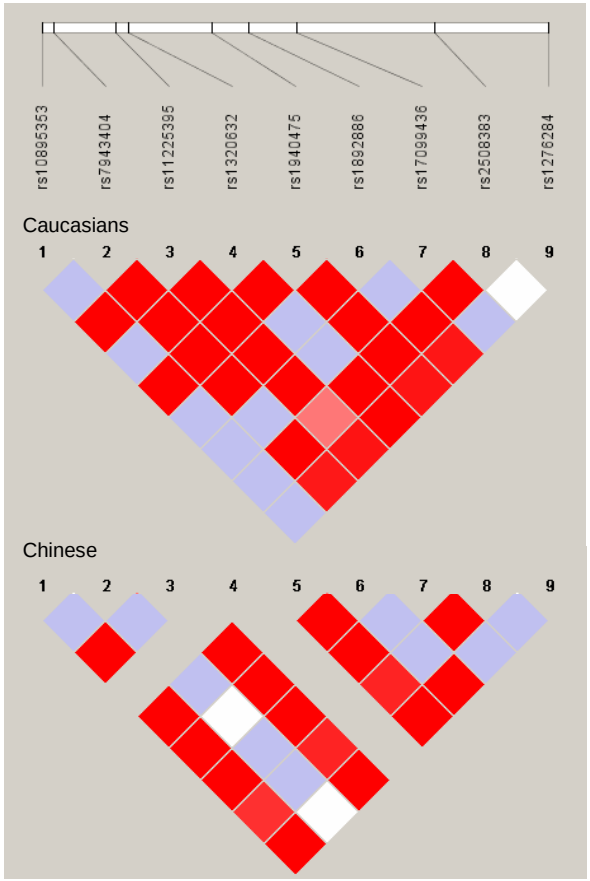
A4.1.1 Genotyping of the Shanghai Breast Cancer Study

Shanghai Breast Cancer subjects were genotyped for the rs11225395 SNP, also using the Applied Biosystems Taqman SNP Genotyping System (C___1366493_10).

A4.1.2 Evaluation of the rs11225395 SNP in the Shanghai Breast Cancer Study cohort

The rs11225395 SNP was chosen as the most suitable candidate for further evaluation by collaborators in America in the Shanghai Breast Cancer Study. The reasons being that this SNP, in the Leuven breast cancer study, showed the strongest association with lymph node metastasis, and is in linkage disequilibrium with the other 3 associated SNPs (rs1940475, rs1892886, and rs1276284, Fig A1). It also tags the four major haplotypes with C allele tagging haplotypes 1 and 3, which carry an increased risk of metastasis, and the T allele tagging haplotypes 2 and 4 which carry a reduced risk of metastasis.

Figure A1. LD plot, showing the LD patterns of the MMP8 SNPs in Caucasian and Chinese populations



The results of their analysis of the rs11225395 SNP within the Shanghai study population are presented in tables A1 and A2, the genotypes stratified by TMN stage and cancer relapse status are shown in table A1. In all patients there was a trend towards a reduced rate of relapse for carriers of the T allele, though it was not significant. Patients with early-stage cancer (TNM stage 0-II) carrying the minor allele (T) had a lower rate of relapse versus those homozygous for the C allele in patients with early-stage cancer ($p = 0.04$, Table A1).

Table A1**. MMP8 SNP rs11225395 in relation to TNM stage and breast cancer relapse in the Shanghai Breast Cancer Study**

	TNM stage					Cancer relapse in all patients			Cancer relapse in early stage (0-II) patients		
Genotype	0-I N (%)	IIa N (%)	IIb N (%)	III-IV N (%)	P	No relapse N (%)	Relapse N (%)	P	No relapse N (%)	Relapse N (%)	P
CC	100 (36.0)	145 (37.3)	94 (39.3)	47 (38.8)		303 (73.9)	107 (26.1)		256 (76.4)	79 (23.6)	
CT + TT	178 (64.0)	244 (62.7)	145 (60.7)	74 (61.2)	**0.87***	514 (78.1)	144 (21.9)	**0.11***	451 (82.2)	98 (17.9)	**0.04***
CT	143 (51.4)	195 (50.1)	108 (45.2)	54 (44.6)	**0.69†**	408 (79.2)	107 (20.8)	**0.13†**	357 (82.3)	77 (17.7)	**0.12†**
TT	35 (12.6)	49 (12.6)	37 (15.5)	20 (16.5)		106 (74.1)	37 (25.9)		94 (81.7)	21 (18.3)	

* P value for the comparison of CT + TT versus CC

† P value for trend test comparing CC, CT and TT

The genotype distribution in relation to survival is shown in table A2. Survival rates were higher in T allele carriers versus C homozygotes; this was again more evident amongst patients with an early-stage cancer (TNM stage 0-II). The adjusted hazard ratios (HR) for T allele carriers were 0.7 (95% CI, 0.5–1.0; P = 0.02) for overall survival and 0.7 (95% CI, 0.5–0.9; P = 0.02) for disease-free (cancer recurrence/metastasis free) survival.

Table A2**. MMP8 SNP rs11225395 in relation to survival among breast cancer patients in the Shanghai Breast Cancer Study**

Population	Genotype	Overall survival				Disease-free survival			
		cases	events	HR (95% CI)	P	cases	events	HR (95% CI)	P
All women									
	CC	415	98	1.0 (reference)		410	127	1.0 (reference)	
	CT + TT	681	139	0.9 (0.7,1.1)	0.23*	658	172	0.8 (0.7,1.0)	0.09*
	CT	530	109	0.9 (0.7,1.1)	0.25†	515	131	0.8 (0.6,1.0)	0.24†
	TT	151	30	0.8 (0.5,1.2)		143	41	0.9 (0.6,1.3)	
TNM 0-II									
	CC	339	70	1.0 (reference)		335	93	1.0 (reference)	
	CT + TT	567	83	0.7 (0.5,1.0)	0.02*	549	115	0.7 (0.5,0.9)	0.02*
	CT	446	69	0.7 (0.5,1.0)	0.01†	434	91	0.7 (0.5,1.0)	0.03†
	TT	121	14	0.5 (0.3,1.0)		115	24	0.7 (0.5,1.1)	
TNM III-IV									
	CC	47	19	1.0 (reference)		46	24	1.0 (reference)	
	CT + TT	74	40	1.5 (0.9,2.6)	0.14*	70	42	1.3 (0.8,2.2)	0.31*
	CT	54	30	1.6 (0.9,2.8)	0.26†	52	30	1.2 (0.7,2.1)	0.22†
	TT	20	10	1.4 (0.6,3.0)		18	12	1.5 (0.8,3.1)	

* P value for the comparison of CT + TT versus CC

† P value for trend test comparing CC, CT, and TT

A graphical representation of survival over time, the Kaplan-Meier survival curves (Fig. A2) show a slight increase in overall and disease free survival in carriers of the T allele (48).

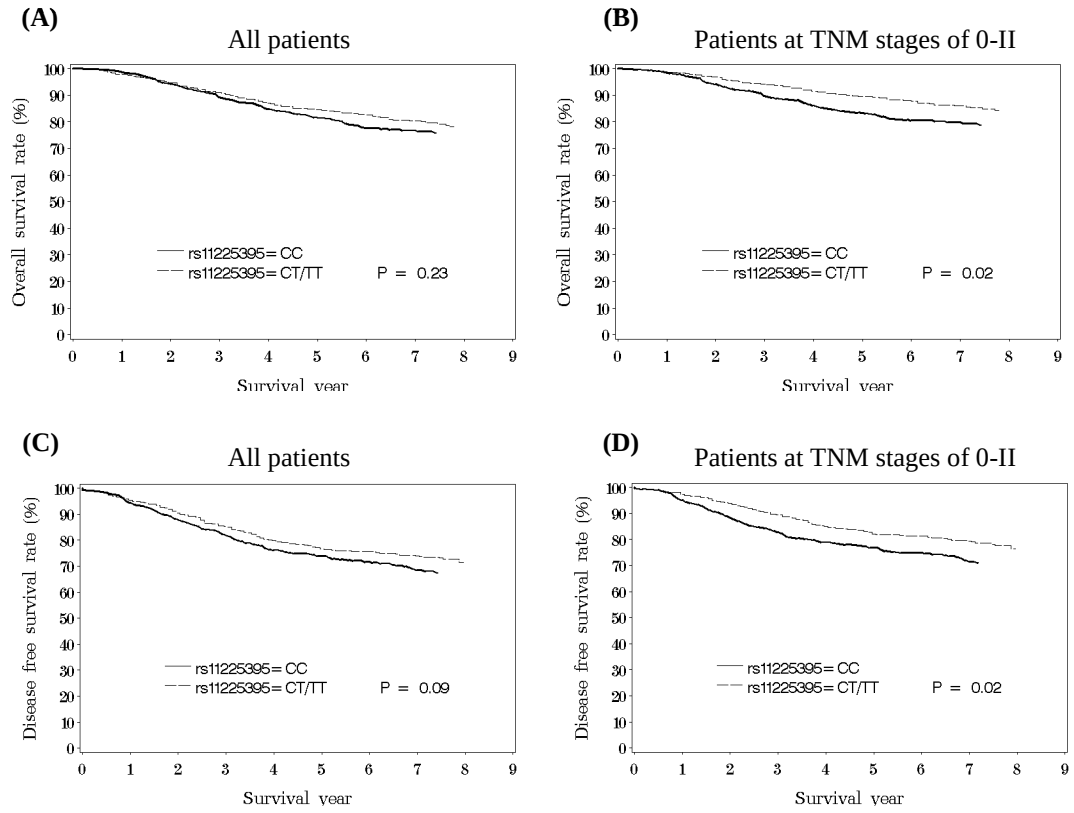


Figure A2. Overall and disease-free survival of breast cancer patients after diagnosis stratified by genotypes of SNP rs11225395. (**A**), overall survival among all women; (**B**), overall survival among women at TNM stages of 0 to II; (**C**), disease-free survival among all women; (**D**), disease-free survival among women at TNM stages of 0 to II.

A4.2 MMP8 promoter SNP rs11225395 EMSA using nuclear protein extract from cancer cells

Electrophoretic Mobility Shift Assay (EMSA); double stranded biotin labelled oligonucleotide probes were used for each rs11225395 allele (C: 5'-CCATGCAGAGCCTATAGTAGCTCC-3' and T: 5-CCATGCAGAGCTTATAGGTAGCTCC-3'). The probes were incubated MDA-MB-231 cell nuclear protein extract, the resulting protein DNA complexes were separated using poly-acrylamide gel electrophoresis (PAGE); detection was with a LightShift Chemiluminescent EMSA kit (Pierce Biotechnology).

A4.2.1 EMSA Results

One of the principal mechanisms by which SNPs in the promoter region of genes can functionally effect expression is via interaction with nuclear proteins. The C allele probe did not form any detectable DNA-protein complexes (Fig A3 lane 2). There were 3 DNA-protein complexes detected by incubation of nuclear extract with the probe corresponding to the T allele (Fig A3 lane 9). Large amounts of unlabelled T oligonucleotide probe, acting as a competitor, caused the DNA-protein complex bands to be diminished (Fig. A3, lanes 12 and 13). The DNA-protein complex bands were unaffected by the presence of large amounts of an unlabelled C allele oligonucleotide (Fig. A3, lanes 10 and 11) or large amounts of a non-specific oligonucleotide competitor (Fig. A3, lane 14).

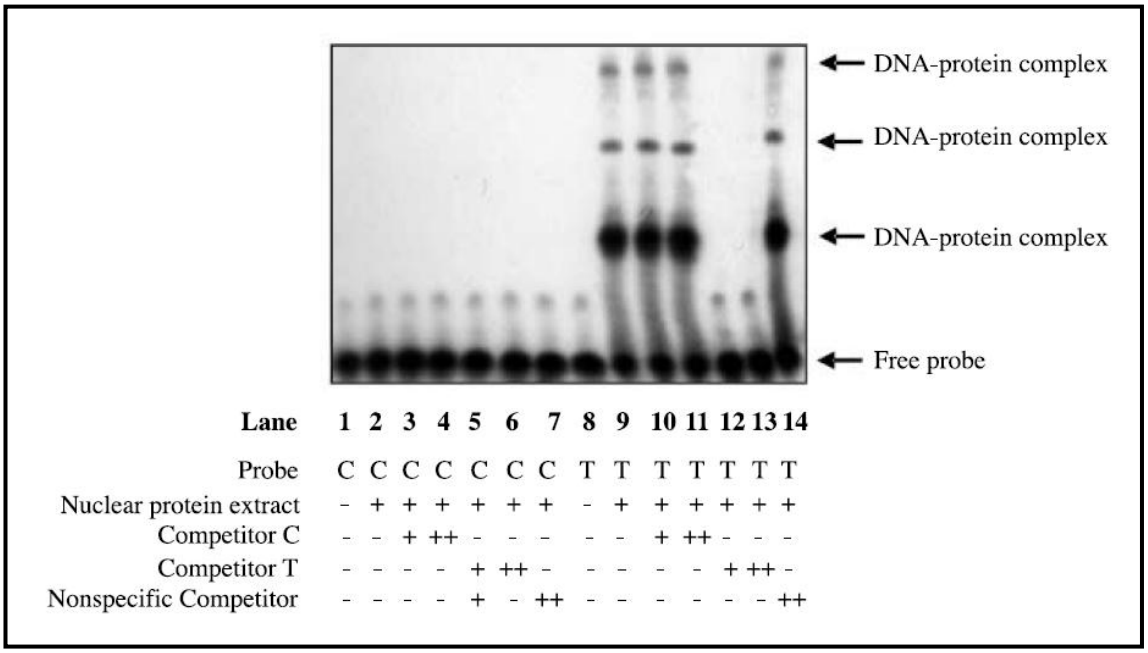


Figure A3. Representative results of electrophoretic mobility shift assays. Nuclear protein extracts from MDA-MB-231 breast cancer cells were incubated with biotin-labelled probes corresponding to the C allele (lanes 1–7) or the T allele (lanes 8–14) of the -799C>T SNP (rs11225395) in the absence or presence of competitors. Lanes 1 and 8, no nuclear protein extract; lane 2 and 9, no competitor; lanes 3 and 10, competitor C in 20-fold molar excess (+); lanes 4 and 11, competitor C in 50-fold molar excess (++); lanes 5 and 12, competitor T in 20-fold molar excess (+); lanes 6 and 13, competitor T in 50-fold molar excess (++); lanes 7 and 14, nonspecific competitor in 50-fold molar excess (++).

A4.3 Functional analysis of MMP8 non-synonymous SNP rs1940475

A full length MMP8 cDNA sequence containing the C (Glu87) allele was purchased (OriGen, Rockville, MD). Site directed mutagenesis was then used to create a cDNA sequence containing the T (Lys87) allele. The cDNAs were cloned into a TOPO expression vector (Invitrogen). Glu87 and Lys87 forms of MMP8 Zymogens were synthesised using the TNT quick coupled transcription/translation system (Promega). Both the zymogens were incubated with or without 1.5 mM APMA for 1 hour at 37°C, before undergoing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Detection was with a monoclonal anti-MMP8 antibody (R&D Systems), and Western blot analysis. Band intensities of the latent form (60kDa and 70kDa) and active form (40kDa and 45kDa) were quantified with the use of Image-pro software.

A4.3.1 The T allele of rs1940475 is less amenable to activation

The rs1940475 polymorphism is a non-synonymous substitution effecting a change in the amino acid sequence of the protein from a glutamic acid residue to a lysine residue at position 87. This residue is in close proximity to the cysteine switch, within the propeptide domain, a crucial mechanism in the conversion of the latent MMP8 zymogen into its active lytic form (253). In vitro Western blot analysis was carried out to investigate whether this SNP has an effect on MMP8 activation. It was found that the MMP8 Lys87 zymogen, coded for by the T allele is less amenable to activation than the Glu87 zymogen (C allele) (Figure A4).

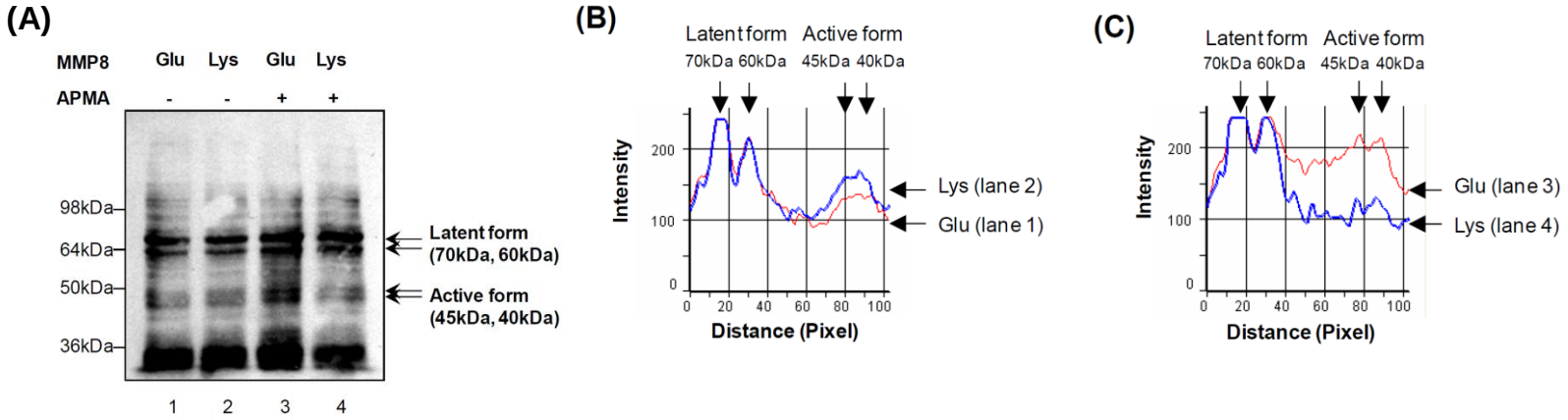


Figure A4**.** Western blot analysis showing that MMP8 zymogen with Lys87 is less amenable to activation than MMP8 zymogen with Glu87 (**A**) Image of Western blot analysis. MMP8 zymogens with Glu87 or Lys87 were incubated in the presence or absence of the activator 4-aminophenylmercuric acetate (APMA) and then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by Western blot analysis with the use of a monoclonal anti-MMP8 antibody that recognizes both active and latent MMP8.
(**B**) and (**C**) Curves represent intensities of the bands of latent (60kDa, and 70kDa likely representing a glycosylation form) and active (40kDa, and 45kDa likely representing a glycosylation form) MMP8 with Glu87 (red lines) or Lys87 (blue lines). (**B**) shows that in the absence of the activator APMA, the intensities of the 40kDa and 45kDa bands (active MMP8) are similar between MMP8 with Glu87 (red line, representing intensities of bands in lane 1 in Western blot image) and MMP8 Lys87 (blue line, representing intensities of bands in lane 2 in Western blot image). (**C**) shows that in the presence of APMA, the intensity of the 40kDa and 45kDa bands (active MMP8) produced by MMP8 with Lys87
(blue line, representing intensities of bands in lane 4 in Western blot image) are lower than those produced by MMP8 with Glu87 (red line, representing intensities of bands in lane 4 in Western blot image), indicating that MMP8 zymogen with Lys87 is less amenable to activation than the zymogen with Glu87.

A4.4 Flow cytometry

Mouse endothelial cells (C166) were incubated with Ang I that had been subjected to cleavage by MMP8 or uncleaved Ang I, in the absence or presence of the Ang II type 1 receptor (AT_1R) antagonist losartan and the Ang II type 2 receptor (AT_2R) antagonist PD123319, and then subjected to flow cytometric analysis of VCAM-1 expression.

A4.4.1. Angiotensin II induces VCAM-1 over a wide range of concentrations

The atherogenic properties of Ang II can partly be ascribed its induction of adhesion molecules (201, 203); VCAM-1 in particular, expressed on the vascular endothelium, is known to play a role in the formation of atherosclerotic lesions (227, 228, 254-256). In vitro and FACS experiments confirmed that VCAM-1 is induced by a wide range of Ang II concentrations in endothelial cells, Figure A5.

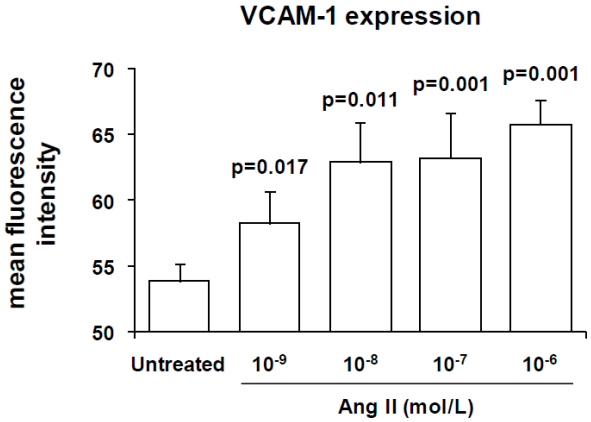


Figure A5. Chart showing that Ang II in a wide range of concentration increases VCAM-1 expression in cultured endothelial cells. Shown in chart are mean fluorescence intensities (and SEM) in flow cytometric analysis. P-values shown are for comparison between Ang II treated cells and untreated cells.

A.4.4.2 Products of Ang I cleavage by MMP8 also induce VCAM-1 expression in endothelial cells

It was also found also found that VCAM-1 expression was increased in endothelial cells when they were incubated with products of Ang I cleavage by MMP8 compared to endothelial cells treated with Ang I (p=0.002; fig A6 A and B). This increase was no longer seen on repeating the same experiments this time in the presence of angiotensin receptor I (AT_1R) antagonist Losartan and angiotensin receptor II (AT_2R) antagonist PD123319 (p=0.937; fig A6 C and D).

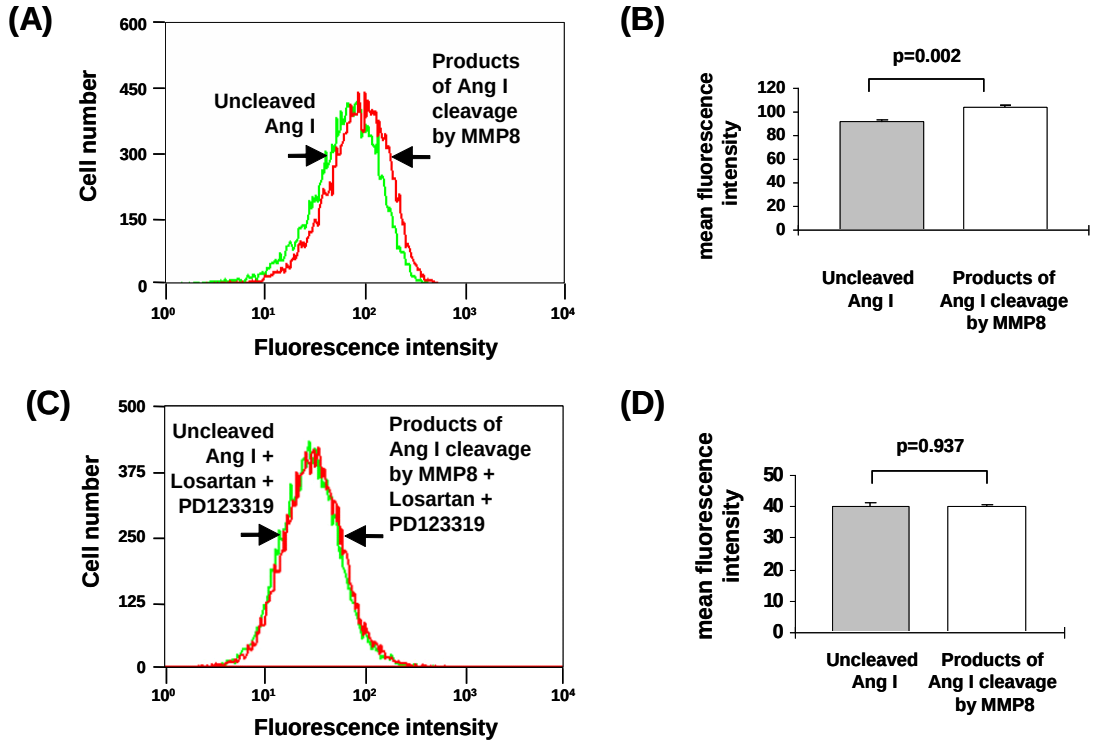


Figure A6. Products of Ang I cleavage by MMP8 increase VCAM-1 expression in endothelial cells. Panels A and B: representative plot (**A**) and mean fluorescence intensity (**B**) in flow cytometric analyses of VCAM-1 expression on endothelial cells incubated with uncleaved Ang I or with products of Ang I cleavage by MMP8. Panels C and D: representative plot (**C**) and mean fluorescence intensity (**D**) in flow cytometric analyses of VCAM-1 expression on endothelial cells incubated with uncleaved Ang I or with products of Ang I cleavage by MMP8, in the presence of the AT_1R antagonist losartan and the AT_2R antagonist PD123319. Error bars in the column charts are standard error of mean.

A4.5 Real-time reverse-transcriptase-PCR in MMP8$^{-/-}$ apoE$^{-/-}$ mice & controls

Real-time RT-PCR was used to quantify the expression of other murine MMPs in the two groups. RNA was extracted from aortas and reverse transcribed into cDNA. The MMPs quantified, in duplicate, were MMP2, MMP9, MMP13 and MMP14. GAPDH was used as the house keeping reference; detection was with Sybr Green on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). PCR primers used are shown in the Table A3.

Table A3**. Primers used to quantify expression of murine MMPs**

Gene	Forward primer	Reverse primer
MMP2	5'-GTCGCCCCTAAAACAGACAA-3'	5'-GGTCTCGATGGTGTTCTGGT-3'
MMP9	5'-CGTCGTGATCCCCACTTACT-3'	5'-AACACACAGGGTTTGCCTTC-3'
MMP13	5'-GCCCTGATGTTTCCCATCTA-3'	5'-TTTTGGGATGCTTAGGGTTG-3'
MMP14	5'-GGATACCCAATGCCCATTGGCCA-3'	5'-CCATTGGGCATCCAGAAGAGAGC-3'
GAPDH	5'-TGACGTGCCGCCTGGAGAAAC-3'	5'-CCGGCATCGAAGGTGGAAGAGT-3'

Gene expression data was calculated using the ΔΔCT method, standardised against GAPDH reference house-keeping gene values.

A.4.5.1 Expression levels of other murine MMPs

There were no differences between the 2 groups when measuring the expression levels of other mouse MMPs including MMP2, MMP3, MMP13, and MMP14 (p=0.108, p=0.611, p=0.509, and p=0.318, respectively; Fig A7).

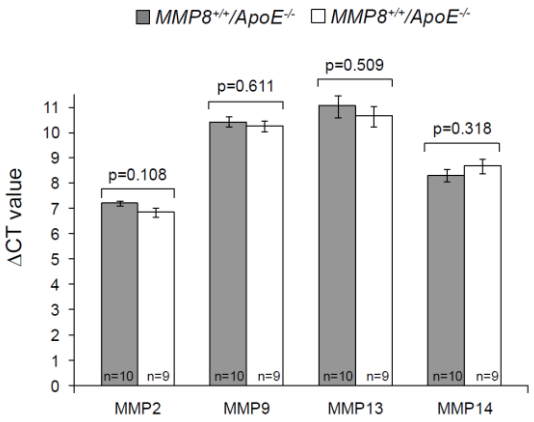


Figure A7. RT-PCR analysis of MMP2, MMP9, MMP13 and MMP14 and GAPDH. Real-time RT-PCR data were analysed using the ΔΔCT method, standardized against GAPDH reference house-keeping gene values. Data shown in chart are mean MMP CT values after being subtracted by GAPDH CT values. Error bars represent standard error of mean.

A4.6 Immunohistochemical characterisation of atherosclerotic lesions in murine study

Immunohistochemical analysis of Ang II in the atherosclerotic lesions was done utilising an immunoperoxidase system (Vectastain Elite ABC kit, Vector Laboratories) in conjunction with a rabbit anti-angiotensin II antibody (Phoenix Pharmaceuticals) and a rabbit IgG isotype negative control. Immunohistochemical analysis, with antibodies against the relevant markers for macrophages (Mac-1, BD Biosciences Pharmingen), smooth muscle cells (α-actin, Sigma-Aldrich), collagen type I (Abcam), and VCAM-1 (BD Biosciences Pharmingen), was used to assess the other contents of atherosclerotic lesions in frozen aortic root sections.

A4.6.1 Immunohistochemical characterisation results

There were reduced Ang II levels in the atherosclerotic lesions of MMP8-/-ApoE-/- mice compared the lesions of wild type MMP8 mice (p=0.002; fig A8 A). There was a 70% reduction (p=0.002, Figure A8 B) of macrophages within the lesions of double knockout mice in comparison with the lesions of the controls. Oil red O staining showed that the atherosclerotic lesions in the double knockout mice had a lower lipid content (reduced by 47%, p=0.001, Figure A8 C). Fewer smooth muscle cells were observed in the lesions of the MMP8 deficient mice than lesions in the controls, although the difference did not reach statistical significance (p=0.057, Figure A8 D). Collagen content was higher in the lesions in the double knockouts than lesions in the controls (differing by 27%, p=0.034, Figure A8 E).

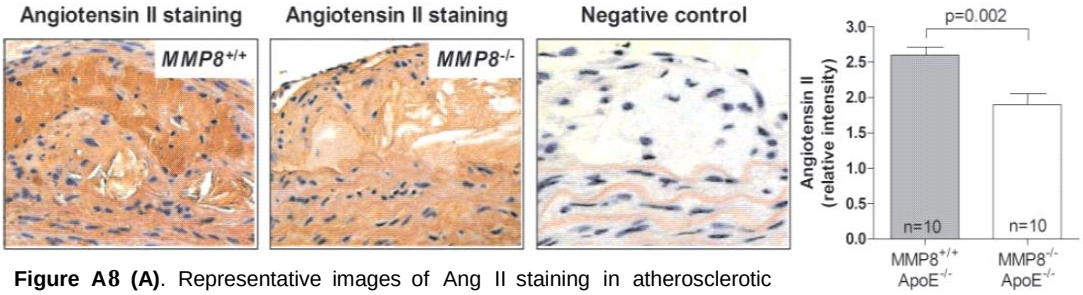
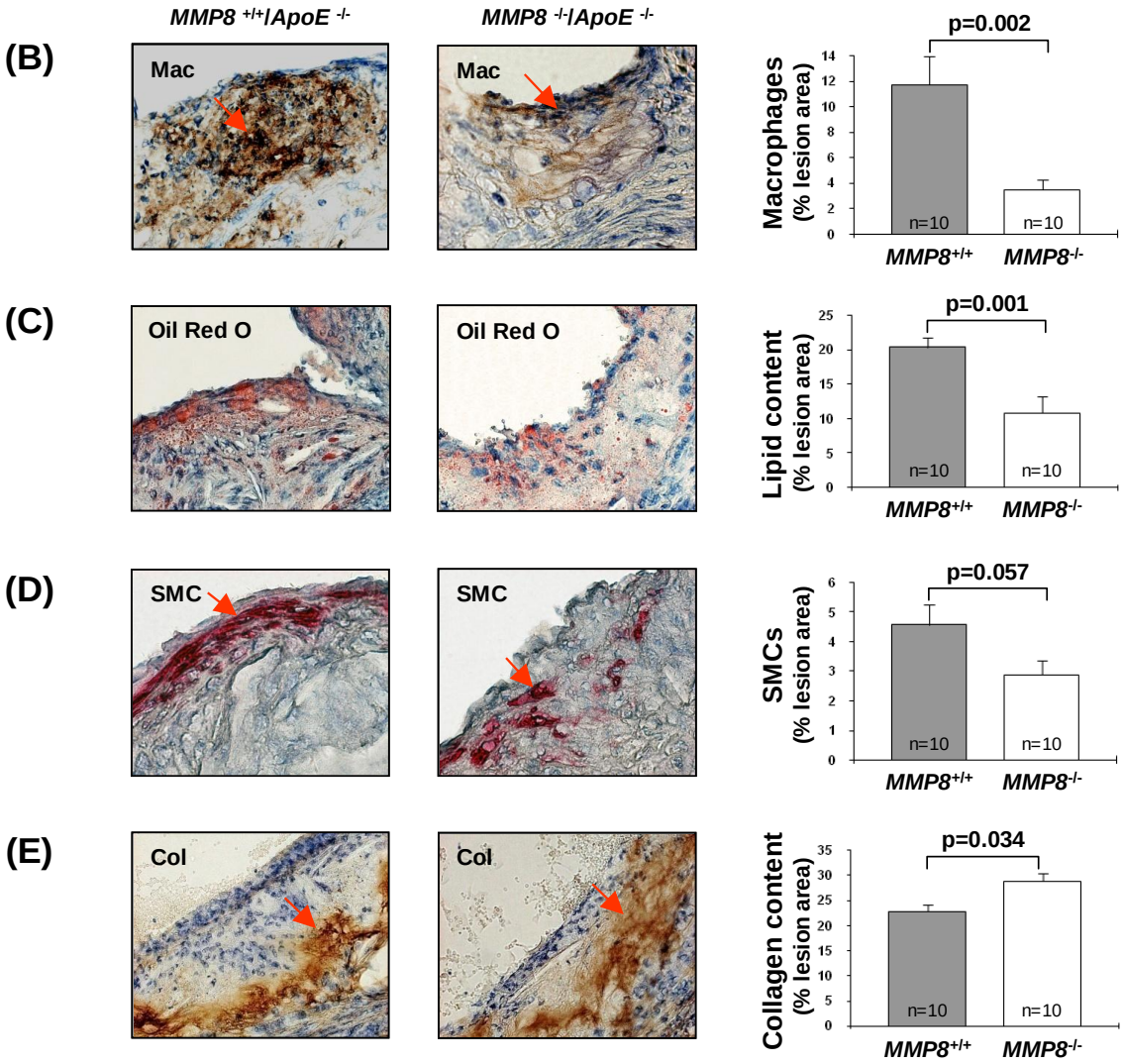


Figure A8 (A). Representative images of Ang II staining in atherosclerotic lesions in MMP8-/-apoE-/- mice and MMP8+/+ApoE-/- mice, respectively, and column chart showing lower relative intensity of Ang II staining in lesions in MMP8-/-ApoE-/- mice than in MMP8+/+ApoE-/- mice. Data presented as means and standard error of mean



A8 continued. Reduced atherosclerosis in MMP8-/-apoE-/- mice. Panels B, C, D and E: representative images of immunostaining of macrophages (**B**), lipids (**C**), smooth muscle cells (**D**) and collagen (**E**) in aortic root atherosclerotic lesions of MMP8+/+apoE-/- and MMP8-/-apoE-/- mice, and charts showing percentages of mean macrophage, lipid, smooth muscle cell, and collagen contents in the two groups of mice. Data presented are mean and standard error of mean.

A4.7 Murine Blood pressure measurement

Blood pressure was measured in conscious mice using a volume pressure recording sensor and occlusion tail cuff (Coda2, Kent Scientific, USA).

A.4.7.1 Lower blood pressure in double knockout mice

Blood pressure was lower in knockout animals, this is consistent with their reduced levels of the potent vasoconstrictor Ang II (P=0.017 for systolic and P=0.023 for diastolic blood pressure; Figure A9).

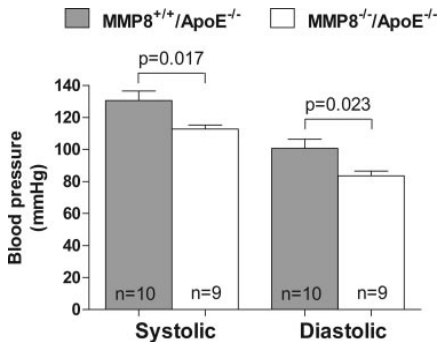


Figure A9. Reduced blood pressure in MMP8-/-ApoE-/- mice. Chart showing lower systolic and diastolic blood pressure in MMP8-/-ApoE-/- mice than in MMP8+/+ApoE-/- mice. Data shown are means and ±SEM.

A4.7.2 Immunohistochemical analysis of VCAM-1

Immunohistochemical analyses showed that VCAM-1 expression was much reduced in the lesions and endothelial cells of double knockout animals compared to the controls (p=0.015 and 0.005 respectively; fig A10 B, C).

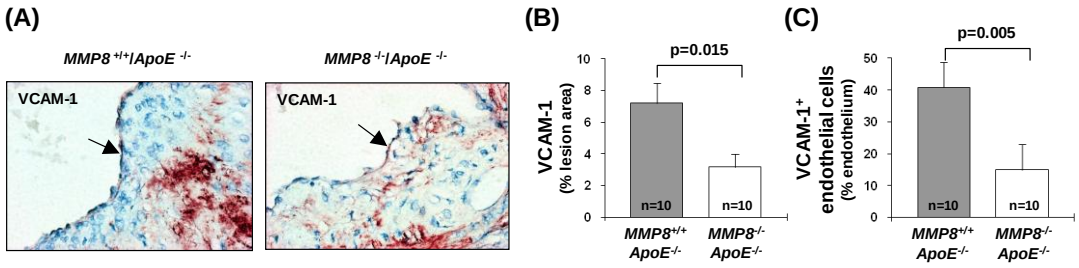


Figure A10. Reduced VCAM-1 expression in MMP8$^{-/-}$/apoE$^{-/-}$ mice compared with MMP8$^{+/+}$/apoE$^{-/-}$ mice
Panels A, B and C show results of immunohistochemical analyses of VCAM-1 in aortic atherosclerotic lesions, (A), representative images; (B), mean VCAM-1 staining in lesions; (C), mean VCAM-1 staining in the endothelial layer. Error bars in the column charts are standard error of mean.

A4.8 Intravital microscopy

Mesenteries of anaesthetised mice were superfused with bicarbonate buffered solution at 37°C at a rate of 2 ml/min. Cell rolling was determined by counting the number of leukocytes rolling per minute in each vessel. Cell adhesion was quantified by counting, for each vessel, the number of adherent leukocytes in a 100 µm length.

A4.8.1 Leukocyte rolling reduced in double knockout mice

Leukocyte rolling and adhesion on the vascular endothelium was shown to be reduced by approximately 50% in double knockout mice compared to MMP8 wild type controls, as measured by intravital microscopy (p=0.049 and p=0.009 respectively; Figure A11).

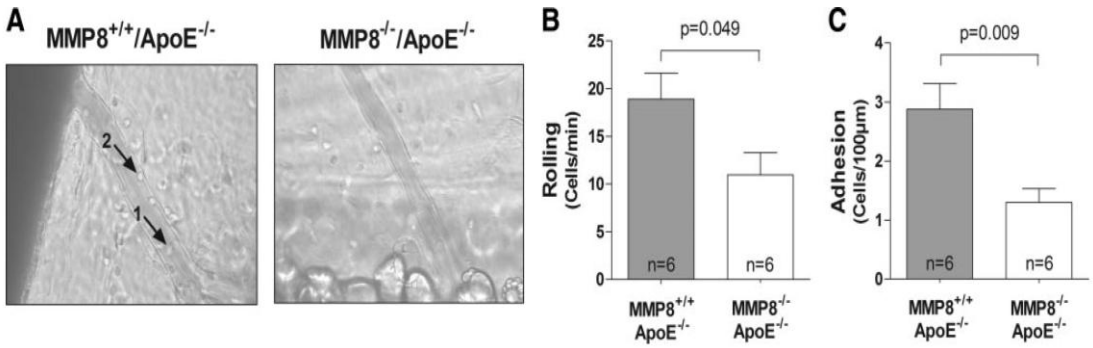


Figure A11. Reduced leukocyte recruitment in MMP8-/-apoE-/- mice. A, Intravital microscopic images showing leukocyte rolling (arrow point 1) and adhesion (arrow point 2) on mesenteric postcapillary venules in vivo. B and C, Diagrams showing that leukocyte rolling and adhesion is reduced in MMP8-/-apoE-/- mice compared with MMP8+/+apoE-/-mice. Data are presented as the means and standard error of mean.